INHIBITION OF NITRIC OXIDE SIGNALING BY MELATONIN IN PORCINE CORONARY ARTERIES

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The Supervisory Committee certifies that this *disquisition* complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

This research represents an effort to bridge a critical gap in the understanding of the local effects of melatonin on the coronary circulation. The first objective was designed to identify the specific melatonin receptor mediating the inhibitory effect of melatonin on nitric oxide (NO)-induced relaxation of porcine coronary arteries. Based on current data, one can conclude that the melatonin (MT) type-2 receptor mediates the inhibitory effect of melatonin on coronary arterial relaxation induced by either exogenous or endogenous NO.

I have also tested the hypothesis that melatonin inhibits the NO-induced increase in cGMP levels by increasing the degradation of cGMP by cGMP specific phosphodiesterase (PDE). The results suggest that in coronary arteries melatonin acts via MT_2-receptors and stimulates the PKG1-dependent PDE5 phosphorylation, resulting in decreased cyclic GMP accumulation in response to NO and impaired NO-induced vasorelaxation.

It was further tested whether the impairment of NO-induced relaxation of porcine coronary arteries by melatonin involves inhibition of NO-activated BK_{Ca} channel activity in porcine coronary smooth muscle cells. Findings from these studies provide evidence that the impairment of coronary artery relaxation in response to NO by melatonin involves MT_2-receptor coupled inhibition of NO-activated BK_{Ca} currents.

The results of these studies have improved our understanding of the role of melatonin in regulation of local coronary vascular tone. Inasmuch as the NO pathway is a critical regulator of coronary arterial smooth muscle tone, inhibition of this pathway may contribute to the mechanisms by which melatonin influences coronary arterial function in health and disease.

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At the end, I wish to thank my parents, my lovely wife and almighty god for giving me the strength and motivation.

DEDICATION

To my parents, my lovely wife and almighty god!

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF SCHEMES

Schemes **Page**

LIST OF ABBREVIATIONS

4-AP……4-Aminopyridine

4P-PDOT……...4-Phenyl-2-propionamidotetralin

5-MCA-NAT……..……5-methoxycarbonylamino-N-acetyltryptamine

8-Br-cGMP……8-Bromo-cGMP

8-Br-PET-CGMPS……....2-Bromo-3,4-dihydro-3-[3,5-O-[(R)-mercaptophosphinylidene]-β-D-ribofuranosyl]-6-pheny-9H-Imidazo[1,2-a]purin-9-one sodium salt

APS……..Ammonium Persulfate

BCA……...Bicinchoninic acid

BK_{Ca}……Large conductance, calcium-activated potassium channel

BSA……..Bovine serum albumin

Ca^{2+}……...Calcium

$CaCl_2$……..Calcium chloride

cGMP……...Guanosine 3',5'-cyclic monophosphate

C_m……..……Membrane capacitance

CO_2……Carbon dioxide

DDT……..DL-Dithiothreitol

DMSO……..Dimethylsulfoxide

EC_{50}……...Concentration necessary to produce 50% of the maximal response

ELISA……Enzyme-linked immunosorbent assay

E_{max}……Maximal decrease in tension

GTP……...Guanosine triphosphate

HEPES……4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)

HRP……...Horseradish peroxidase

K……..……Potassium

KCl……..…….Potassium chloride

KH_2PO_4……Potassium dihydrogen phosphate

K_V……Voltage gated potassium channels

$MgSO_4$……Magnesium Sulfate

NaCl……Sodium chloride

$NaHCO_3$……Sodium bicrabonate

NaOH……Sodium hydroxide

NO……Nitric oxide

NOS……Nitric oxide synthase

O_2……Oxygen

ODQ……1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one

pA/pF……picoamperes per picofarad

PBS……Phosphate buffered saline

PBST……Phosphate buffered saline with 0.1 % V/V Tween 20

pD_2……-log (M) EC_{50}

PDE……Phosphodiesterase

P-PDE……Phosphorylated phosphodiesterase

PKG……Protein kinase G

PKC……Protein kinase C

PSS……Physiological salt solution

PVDF……Polyvinylidene difluoride

SDS-PAGE……sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM……Standard error of mean

sGC……Soluble guanylyl cyclase

SNP……Sodium nitroprusside

STOC……Spontaneous Transient Outward Currents

TEMED……N,N,N,N-Tetramethylethylenediamine

UK 14,304……[5-Bromo-6-(2-imidazolin-2-lamino)quinoxaline]

U44619……9,11-dideoxy-11a,9a-epoxymethano-prostaglandin $F_{2\alpha}$.

CHAPTER 1. INTRODUCTION

1.1 Melatonin physiology

1.1.1 Melatonin: discovery, structure and biosynthesis

Since its discovery in 1958 by Lerner and colleagues, considerable progress has been made in understanding the neurobiology of melatonin (Lerner et al., 1958). In 1963 Wurtman et al. recognized that its rhythmic synthesis is controlled by light (Wurtman et al., 1963) and in 1973, Moore proved that the suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the “master oscillator” underlying most of the body’s 24-h rhythmicity, including the synthesis of melatonin (Moore, 1973). Melatonin is a neurohormone and acts as an endogenous transducer of photoperiodic information. Melatonin is primarily synthesized and released from the pineal gland in a circadian manner with melatonin levels peaking at night and falling to near undetectable levels in day time. In addition, evidence has accumulated that melatonin is produced in several peripheral organs including bone marrow, brain, retina, gastrointestinal tract, skin and particularly relevant to this study, platelets, leukocytes, mast cells and endothelial cells (Bubenik, 2002; Tan et al., 2003; Pandi-Perumal et al., 2008a; Slominski et al., 2008). In these tissues melatonin may act as an autocoid or paracoid signal thereby influencing local physiology (Pandi-Perumal et al., 2008b; Slominski et al., 2012).

Melatonin is a small lipophilic molecule (MW: 232), derived from the amino acid tryptophan, an essential amino acid. Prerequisite for the formation of melatonin is an active uptake of the dietary tryptophan from the circulation into the pineal gland (Pardridge, 1981). Tryptophan is transformed into 5-hydroxytryptophan (5HTP) by tryptophan-5-hydroxylase (Lovenberg et al., 1967), a mitochondrial enzyme present in high amounts in the pineal, whose activity is two-to-three fold increased at night in rats (Sitaram and Lees, 1978). 5HTP is decarboxylated to 5-hydroxytryptamine (5HT, serotonin) by the cytoplasmic enzyme, aromatic amino acid decarboxylase (Lovenberg et al., 1962), a constitutively active enzyme with little daily variation in the pineal.

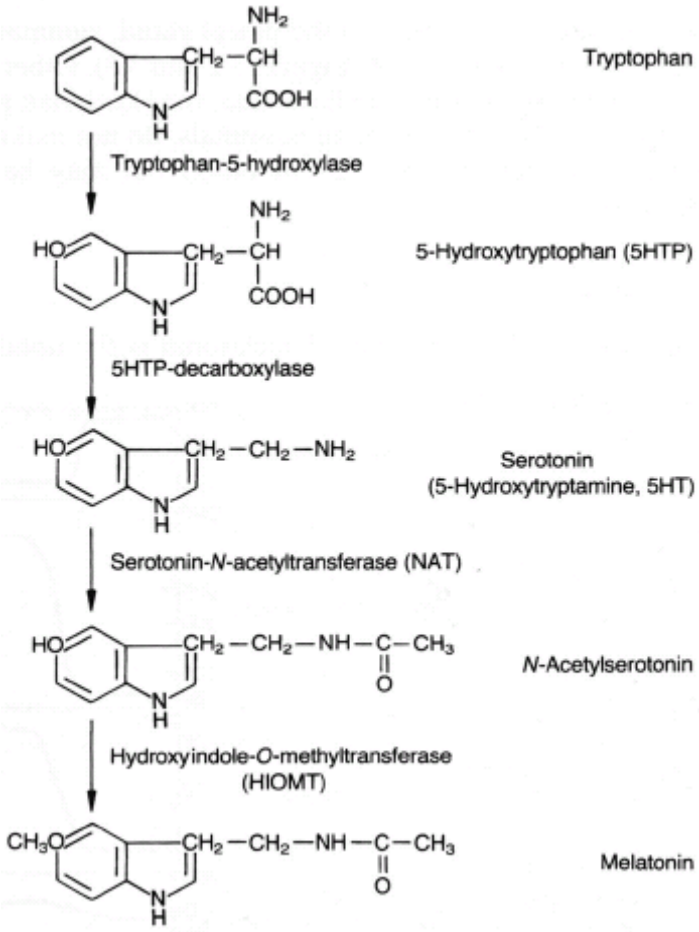


Figure 1. The chemical structure of melatonin and its biosynthetic pathway from tryptophan.

The cytoplasmic enzyme Arylalkylamine-*N*-acetyltransferase (AA-NAT) completes the next step by *N*-acetylation of serotonin using acetyl co-enzyme A as a cofactor (Weissbach et al., 1960). The enzyme is present in the pineal cytoplasm and also in the retina and is distinguished from the same enzyme in other tissues by its specificity and the remarkable variation in its activity leading to 70 to 100 fold increases in activity at night in the rat pineal (Klein and Weller, 1970). Thus, this enzyme appears to be rate limiting in melatonin synthesis. The final step in the pathway is *O*-methylation of *N*-acetylserotonin (NAS) by hydroxyindole-*O*-methyltransferase (HIOMT) (Weissbach, 1960). This enzyme does not exhibit diurnal variation but has a high constitutive activity (Sugden et al., 1987). Therefore, NAS levels increase at night through altered activity of the

rate-limiting enzyme AA-NAT, and this result in enhanced melatonin synthesis from NAS due to the high constitutive activity of HIOMT (Reiter et al., 1983).

1.1.2 Melatonin receptors

The physiological effects of melatonin are considered to be mediated via specific melatonin receptors. These receptors are distinguishable on the basis of their molecular structures (Reppert et al., 1996), their pharmacological characteristics (Dubocovich et al., 1997) and their chromosomal localization (Slaugenhaupt et al., 1995). Whereas MT_1- and MT_2-receptors belong to the G-protein-coupled family of membrane receptors with 60% overall sequence homology, the *MT_3*-receptor is identified as the enzyme quinone reductase 2, an intracellular melatonin binding site (Nosjean et al., 2000).

1.1.3 General structure and signal transduction mechanism of melatonin receptors

The MT_1 and MT_2-receptors represents classical G-protein coupled receptors (GPCRs) consisting of seven transmembrane domains and three extracellular and intracellular loops each, an extracellular N-terminal domain and an intracellular C-terminal part (Reppert et al., 1995) (Figure 2). On the basis of structural analyses, melatonin receptors represent a distinct group within the large superfamily of GPCRs with the highest similarity to μ-opioid and type-2 somatostatin receptors (Shiu et al., 1996). Melatonin receptors, upon agonist activation, interact with heterotrimeric G-proteins, and serve as a guanine-nucleotide exchange factor (GEF) to promote GDP dissociation and GTP binding and activation (Morgan et al., 1989; Laitinen et al., 1990). This leads to dissociation of the G-protein complexes into an α-subunit and a βγ dimer, which activate several effectors (Barrett et al., 1994).

MT_1 melatonin receptors can couple to a wide variety of G-proteins including $G_{i\alpha 2}$, $G_{i\alpha 3}$ and $G_{\alpha q}$ (Brydon et al., 1999), $G_{\alpha s}$, $G_{\alpha z}$ and $G_{\alpha 16}$ (Ho et al., 2001; Chan et al., 2002). In case of the MT_2 receptor, only coupling to $G_{\alpha i}$ has been reported (Chan et al., 2002). In the SCN, the activation of MT_2-receptors has been linked with the phospholipase C/diacylglycerol signaling pathway

(McArthur et al., 1997; Hunt et al., 2001). The effectors system involved in MT_1 and MT_2-receptor signaling through G-protein coupling includes adenylyl cyclase, phospholipase C, phospholipase A2, potassium channels and possibly guanylyl cyclase and calcium-activated potassium channels (Vanecek, 1998; Dubocovich et al., 2003; New et al., 2003).

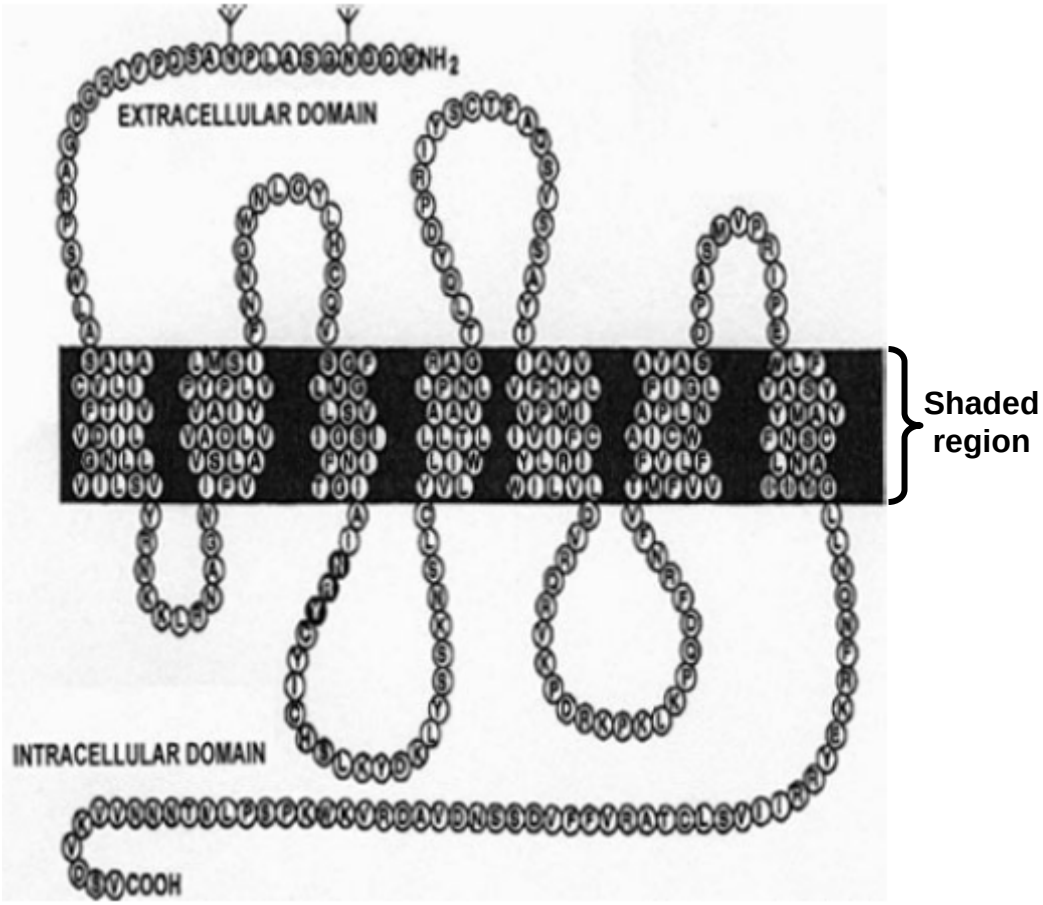


Figure 2. The primary structure and predicted topology of the human MT_2 melatonin receptor. Amino acids in shaded region are identical between MT_2 and the human MT_1 receptor (Reppert et al., 1995). The amino acid homology for the human MT_1 and MT_2-melatonin receptors is approximately 60% overall and 73% within the transmembrane domains (Dubocovich et al., 2010).

1.1.4 Vascular actions of melatonin

Mounting evidence in literature point to a role for melatonin in cardiovascular system (Krause et al., 1999). Previous studies identified the presence of high affinity melatonin binding sites in several vascular beds from a variety of species (Viswanathan et al., 1990; Stankov et al., 1992; Stankov and Fraschini, 1993; Masana et al., 2002) including humans (Ekmekcioglu et al., 2001; Ekmekcioglu et al., 2003) suggesting a role for melatonin in local control of blood vessel diameter. Subsequently, evidence supporting a functional role for melatonin in the vasculature came from studies demonstrating that melatonin causes direct vasoconstriction in certain arteries (Evans et al., 1992; Geary et al., 1997; Ting et al., 1997; Viswanathan et al., 1997) and potentiates contractile responses in others (Krause et al., 1995; Ting et al., 1997; Viswanathan et al., 1997; Yang et al., 2001). Recent studies in our laboratory suggest that melatonin modulates vascular tone by acting on the nitric oxide (NO)/cyclic guanosine 3',5'-cyclic monophosphate (Cyclic GMP) signaling pathway by activating specific melatonin receptors (Yang et al., 2001). However, the exact pharmacological identity of specific melatonin receptor mediating the inhibitory effect of melatonin on NO/cyclic GMP signaling and the molecular mechanism(s) involved is not yet delineated.

NO-induced relaxation of vascular smooth muscle involves activation of soluble guanylyl cyclase (sGC) by NO, increased formation of cyclic GMP from GTP, and activation of cyclic GMP-dependent protein kinase (PKG) (Ignarro and Kadowitz, 1985; Ignarro, 1989). Activated PKG phosphorylates numerous ion channels and pumps, most of which promote a reduction in cytosolic calcium (Lincoln and Cornwell, 1991; Butt et al., 1993). In particular, PKG activates BK_{Ca} channels (Alioua et al., 1998; Fukao et al., 1999), which hyperpolarize the arterial smooth muscle cell membrane thereby causing relaxation (Kannan and Johnson, 1995; Nelson and Quayle, 1995; Yamakage et al., 1996; Zhou et al., 1996). NO is a major endogenous vasodilator produced by the endothelium and impairment of its generation and/or action may lead to sudden localized vasospasm, which may initiate or exacerbate episodes of myocardial ischemia (Maseri et al., 1978; Yang et al., 2001). Thus, a detail understanding of the inhibitory action of melatonin on NO/cyclic

GMP signaling in vasculature is crucial for assessing the potential cardiovascular effects of melatonin/melatonin agonists when used in therapeutic doses.

1.2 Gap in knowledge

Melatonin is a functionally versatile and diverse endogenous molecule (Pandi-Perumal et al., 2006). Currently, melatonin/melatonin agonists are used clinically to treat sleep disorders (Turek and Gillette, 2004; Zlotos, 2005; Delagrange and Boutin, 2006) and their therapeutic utility is being investigated for treating cancer (Mills et al., 2005), Alzheimer's disease (Mayo et al., 2005), Parkinson's disease (Mayo et al., 2005; Sharma et al., 2007), osteoporosis (Cardinali et al., 2003; Reiter et al., 2007) and stroke (Reiter et al., 2003; Reiter et al., 2005). Surprisingly, little is known about the potential effect of melatonin/melatonin agonists on the cardiovascular system and, in particular, the coronary circulation. This lack of understanding of the local effect of melatonin on the coronary circulation represents a critical gap in our knowledge regarding the safe use of melatonin as a chronobiotic (Arendt and Skene, 2005). Recent studies in our laboratory indicate that melatonin exerts a powerful indirect effect on coronary arterial smooth muscle through inhibition of NO-induced relaxation (Yang et al., 2001). Inasmuch as the NO pathway is a critical regulator of coronary arterial smooth muscle tone, inhibition of this pathway may contribute to the mechanisms by which melatonin influences coronary arterial function in health and disease.

The primary objective of the present study is to determine the mechanisms underlying the inhibitory effect of melatonin on NO-signaling. **The central underlying hypothesis for the proposed research is that melatonin inhibits NO-induced relaxation of porcine coronary arteries by inhibiting the NO-induced increases in intracellular cGMP levels and activation of large-conductance, Ca^{2+}-activated K (BK_{Ca}) channels by activating specific melatonin receptors expressed in coronary arteries smooth muscle.**

I set following specific aims to test my central underlying hypothesis and accomplish the overall objective of this application.

1.2.1 Specific aim 1: Identification of specific melatonin receptor subtypes mediating the inhibitory effect of melatonin on NO-induced relaxations of porcine coronary arteries.

Previous studies demonstrated that in isolated porcine coronary arteries, melatonin cause inhibition of NO-induced smooth muscle relaxation by activating specific MT-receptors (Yang et al., 2001). However the exact pharmacological identity of MT-receptor subtypes is not yet established. Thus, the aim of the present study was to identify the specific MT-receptor subtype mediating the inhibitory effect of melatonin on NO-induced relaxation.

1.2.2 Specific aim 2: Effect of melatonin on NO-induced increase in intracellular cyclic GMP levels in coronary arterial smooth muscle.

NO in vascular smooth muscle cells stimulates the formation of cyclic GMP by activating sGC, which in turn causes smooth muscle relaxations. Thus, the aim of the current study was to determine if melatonin impairs the NO-induced relaxations of porcine coronary arteries by inhibiting the NO-induced increase in intracellular cyclic GMP levels in coronary arterial smooth muscle by activating specific melatonin receptors.

1.2.3 Specific aim 3: Role of BK_{Ca} channels in the inhibitory effect of melatonin on NO-induced relaxations of porcine coronary arteries.

Relaxation of vascular smooth muscle cells induced by NO involves the activation of BK_{Ca} channels. Activation of BK_{Ca} channels leads to membrane hyperpolarization and closure of the voltage-dependent Ca^{2+} channels, reduces Ca^{2+} influx, and relaxation (Kannan and Johnson, 1995; Nelson and Quayle, 1995; Yamakage et al., 1996; Zhou et al., 1996). Therefore, the aim of this study is to determine if melatonin impairs the NO-induced relaxations of porcine coronary arteries by inhibiting the NO-activated BK_{Ca} channel activity in porcine coronary artery smooth cells.

The importance of understanding the mechanism of melatonin induced inhibition of NO-cGMP signaling in coronary arteries is threefold. Firstly, knowing the mechanism of coronary action of melatonin will help to understand the local vascular action of melatonin and will contribute to the understanding of physiological role of melatonin in regulation of cardiovascular system. Secondly, the information obtained from this project will help understand the potential role of melatonin in

coronary artery diseases which is known to be associated with dysfunctional NO-cGMP signaling (Brunner et al., 2005; Vanhoutte, 2009). Finally, it is surprising that given the wide-spread and generally unsupervised (i.e. over the counter) use of melatonin (Lewy et al., 2006; Lemoine et al., 2007; Srinivasan et al., 2008; 2009), little is known about the potential effects of melatonin on the cardiovascular system, particularly at supra-physiological concentrations that may result from self-medication. The information obtained may help in assessing the cardiovascular effects and safety of melatonin and novel melatonin receptor selective agonists and antagonists in development.

CHAPTER 2. IDENTIFICATION OF SPECIFIC MELATONIN RECEPTOR SYBTYPES MEDIATING THE INHIBITORY EFFECT OF MELATONIN ON NO-INDUCED RELAXATIONS OF PORCINE CORONARY ARTERIES

2.1 Introduction

Increasing evidence indicates that melatonin, the primary hormone secreted by the pineal gland, regulates blood vessel diameter, thereby impacting arterial blood pressure and local blood flow to organs and tissues (Krause et al., 2000; Pandi-Perumal et al., 2008b). The possibility that melatonin may be involved in local regulation of vascular tone was initially suggested by studies that identified the presence of high affinity melatonin binding sites in several vascular beds (Viswanathan et al., 1990; Stankov et al., 1992; Stankov and Fraschini, 1993). Subsequently, evidence supporting a functional role for melatonin in the vasculature came from studies demonstrating that melatonin causes vasoconstriction in certain arteries (Evans et al., 1992; Geary et al., 1997; Ting et al., 1997; Viswanathan et al., 1997) and vasodilation in others (Satake et al., 1991; Weekley, 1991; Doolen et al., 1998). In addition to these direct actions, previous studies from our laboratory reported that melatonin also exerts a powerful indirect effect on coronary arterial tone by inhibiting smooth muscle relaxation induced by either exogenous or endogenous NO (Yang et al., 2001) by activating specific MT receptor. However the exact pharmacological identity of specific MT receptor mediating the inhibitory effect of melatonin on NO-induced relaxation of coronary arteries in not known.

The presence of specific receptors for melatonin has been demonstrated in arteries from several species, including humans (Ekmekcioglu et al., 2001; Ekmekcioglu et al., 2003). Three distinct melatonin receptor subtypes, termed MT_1-, MT_2, and MT_3-receptors, have been identified and mediate the physiological effects of melatonin (Nosjean et al., 2000; Dubocovich et al., 2003). The goal of the present study was to: (1) detect the expression and tissue localization of the

specific MT receptor protein in porcine coronary artery, and (2) to identify the pharmacological identity of the specific MT receptors mediating the inhibitory effects of melatonin on NO-induced relaxation of isolated porcine coronary arteries.

2.2 Materials and methods

2.2.1 Materials

The following drugs were used: bradykinin, isoproterenol, melatonin, sodium nitroprusside (Sigma Chemical Co., St Louis, MO, USA); luzindole, 5-MCA-NAT (5-methoxy-carbonylamino-N-acetyltryptamine), 4P-PDOT (4-Phenyl-2-propionamidotetralin), UK14,304 (5-Bromo-6-(2-imidazolin-2-ylamino)quinoxaline) (Tocris, Ellisville, MO,USA); and U46619 (Cayman Chemical Company, Ann Arbor, MI, USA). Drug solutions were prepared daily, kept on ice, and protected from light until used. All drugs were dissolved initially in distilled water with the exception of melatonin, 5-MCA-NAT, 4P-PDOT and luzindole, which were dissolved in ethanol before further dilution in distilled water. Drugs were added to the organ chambers in volumes not greater than 0.2 ml (i.e. 0.8 % of the total volume). Drug concentrations are reported as final molar concentration in the organ chamber. The composition of the physiological salt solution was as follows (in mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1. (pH=7.4).

2.2.2 Methods

2.2.2.1 Tissue preparation

Fresh porcine hearts were obtained from a local abbatoir and were immediately immersed in cold physiological salt solution and transported to the laboratory within 30-40 minutes. The left anterior descending coronary artery was dissected free from surrounding myocardium, cleaned of adherent fat and connective tissue, and cut into rings 4–5 mm in length. Four to eight coronary arterial rings were prepared from each heart. In some rings, the endothelium was removed by gently rubbing the intimal surface with a fine forceps.

2.2.2.2 Immunoblot analysis

Coronary arteries with intact endothelium were immediately frozen in liquid nitrogen after preparation. For detection of MT-receptor protein, tissues were homogenized with an IKA® Ultra, Turrax-T8 homogenizer (IKA works. Inc., Wilmington, NC, USA) at 4˚C in lysis buffer, supplemented with a protease and phosphatase inhibitor cocktail (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The tissue homogenates were kept on ice for 10 min and afterwards centrifuged for 10 min at 10,000g. Supernatants were collected for protein determination and Western blotting. Protein concentration was assayed using a Bio-Rad D_c Protein assay kit (Biorad Laboratories, Hercules, CA, USA). Aliquots of supernatants containing equal amounts of protein (100-150 µg) were separated on 12% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins were electrophoretically transferred onto a PVDF membrane (Biorad Laboratories, Hercules, CA, USA). Blots were blocked with 5% nonfat dry milk in phosphate buffered saline (PBS, pH 7.4) and then incubated overnight at 4˚C with a primary antibody specific for MT_1-receptors or MT_2-receptors using a dilution of 1:200 (Santa Cruz Biotechnology Inc., USA). Membranes were washed two times for 15 minutes using PBS Tween-20 (PBST) and incubated with a horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology Inc., USA). To ensure equal loading, the blots were analyzed for β-tubulin expression using an anti-β-tubulin antibody (sc-9935; Santa Cruz Biotechnology). Immunodetection was performed using an enhanced chemiluminescence light detecting kit (Thermo Scientific, Rockford, IL, USA). The images are representative of six different samples.

2.2.2.3 Immunohistochemistry

An immunohistochemical method was used for localizing MT_2-receptors in the coronary artery smooth muscle cell layer. Frozen tissue blocks of porcine coronary artery rings with intact endothelium were sectioned into 8 µm thick cryosections using a cryostat microtome at -20˚C. Sections were transferred to ProbeOn™ Plus microscopic slides (Fisher Scientific, Pittsburgh, PA, USA) for further processing. Sections were fixed with ice-cold acetone and air dried for 30 min at room temperature. Non-specific antibody binding was blocked with 1.5% normal donkey serum in

PBS (pH 7.4) for 1 hr at room temperature. The sections were incubated overnight at 4˚C with a primary antibody specific for MT_2-receptors at 1:50 dilution (Santa Cruz Biotechnology Inc., USA). The sections were then incubated for 1 hr in the secondary antibody solution (1:250 dilutions, Alexa Fluor 488-labeled rabbit anti-goat IgG, Molecular Probes, Eugene, OR, USA). A specific MT_2-receptor antibody blocking peptide (Santa Cruz Biotechnology Inc., USA) was used to control for specificity of the antigen-antibody reaction. The primary antibody (at a 1:50 dilution) was neutralized by conjugating with blocking peptide (5 fold excess by weight), overnight at 4˚C. All dilutions and thorough washes between stages were performed using PBS unless otherwise stated. Sections were drained by blotting with filter paper and a drop of mounting medium (containing an anti-fade reagent) was added to the slides before mounting on a standard cover slip. The images of the sections were obtained using an Olympus confocal laser-scanning microscope. The images were generated using FV300 (v. 4.3) Confocal Software and Adobe Photoshop 5.5.

2.2.2.4 Pharmacologic studies

Coronary arterial rings were suspended in water-jacketed organ chambers filled with 25 ml of physiological salt solution, as previously described (Yang et al., 2001; Tunstall et al., 2011). The organ chamber solution was aerated with a mixture of 95% O_2/5% CO_2 and the temperature was maintained at 37˚C throughout the experiment. Each ring was suspended by means of two fine stainless-steel wire clips passed through the lumen; one clip was anchored inside the organ chamber, the other connected to a force transducer (Model FT03, Grass Instrument Company, Quincy, MA, USA). Isometric tension was measured and recorded on a Grass polygraph. The tissues were stretched progressively to the optimal point of their length–tension relationship, using KCl (20 mM) to generate a standard contractile response. After this procedure, the preparations were allowed to equilibrate at their optimal length for at least 30 min prior to further exposure to any vasoactive substances. The absence or presence of intact endothelium was confirmed in each preparation by the absence or presence of relaxation to the endothelium-dependent vasodilator, bradykinin (10^{-7} M).

Relaxation of coronary arteries was studied in rings contracted with 9, 11-dideoxy-11α,9α-epoxymethano-$PGF_{2\alpha}$ (U46619, 1–3 x 10^{-9} M), a thromboxane A_2-mimetic. After the U46619-induced contraction had reached a stable plateau, relaxation responses to increasing concentrations of sodium nitroprusside (SNP; 10^{-9}–10^{-5} M), the α_2-adrenoceptor agonist, UK14,304 (10^{-9}–10^{-5} M) or β-adrenoceptor agonist, isoproterenol (10^{-9}–10^{-5} M) were obtained in the presence and absence of melatonin (10^{-7} M), which was added to the organ chamber immediately prior to the addition of U46619. To determine the potential role of MT_2-receptors in mediating the inhibitory effects of melatonin on NO-induced relaxation of coronary arteries, the arterial rings were incubated with the MT_2-receptor antagonist 4P-PDOT (10^{-7} M) or luzindole (10^{-7} M) for 30 min prior to exposure to melatonin. These inhibitors remained in contact with the tissues throughout the remainder of the experiment. In a separate series of experiments, concentration-response curves to SNP and UK14,304 were also obtained in the absence and presence of the *MT_3*/NQO2-receptor selective agonist, 5-MCA-NAT (10^{-7} M) which was added to the organ chambers in place of melatonin.

2.2.2.5 Cyclic GMP measurements

An enzyme linked immunosorbent assay was used to measure cGMP levels in porcine coronary arteries. Coronary arterial rings were suspended in water-jacketed organ chambers filled with 25 ml of physiological salt solution and were allowed to equilibrate for at least 1 hr at 37℃. After equilibration, coronary artery rings were treated with SNP (5 min, 10^{-5} M) in the presence and absence of melatonin (5 min, 10^{-7} M). In some experiments the rings were pretreated with 4P-PDOT (20 min, 10^{-7} M) prior to exposure to melatonin and SNP. After drug treatments, rings were frozen in liquid nitrogen and homogenized with an IKA® Ultra, Turrax-T8 homogenizer (IKA works. Inc., Wilmington, NC, USA) at 4℃ in 0.1 N hydrochl oric acid. The tissue homogenates were centrifuged for 10 min at ≥ 600g. The cyclic GMP and total protein content was determined in the supernatant using a direct cyclic GMP enzyme immunoassay kit (Assay design, Ann Arbor, MI, USA) and a Bio-Rad Dc Protein assay kit (Biorad Laboratories, Hercules, CA, USA), respectively. Cyclic GMP levels were expressed as pmol/μg of protein.

2.2.2.6 Data analysis

Relaxation responses are expressed as a percentage of the initial tension induced by U46619. For each vasodilator, both the maximal percent relaxation (E_{max}) and the concentration necessary to produce 50% of its own maximal response (EC_{50}) were determined. The EC_{50} values were converted to the negative logarithms and expressed as -log molar EC_{50} (pD_2). Results are expressed as mean ± S.E. and *n* refers to the number of animals from which blood vessels were taken. Values were compared by Student's *t*-test for paired or unpaired observations, or by analysis of variance and a post hoc Bonferroni's multiple comparison analysis to determine significance between groups, as appropriate. Values were considered to be significantly different when $P < 0.05$.

2.3 Results

2.3.1 Immunoblot analysis

The expression of melatonin receptor protein in porcine coronary arteries was determined by immunoblot analysis. A strong immunoreactive band at 36 kDa corresponding to MT_2-receptors was detected in immunoblots of coronary artery homogenates (Figure 3; lanes 1-3). The MT_2-receptor protein immunoreactivity was abolished when the primary antibody was neutralized with a specific blocking peptide. Since MT_2-receptors are known to be expressed in rat heart (Sanchez-Hidalgo et al., 2009), extracts of this tissue were used as a positive control (Figure 3; lane 5). By contrast, there was no evidence for MT_1- receptor and *MT_3*/NQO2 melatonin binding site expression in porcine coronary arteries.

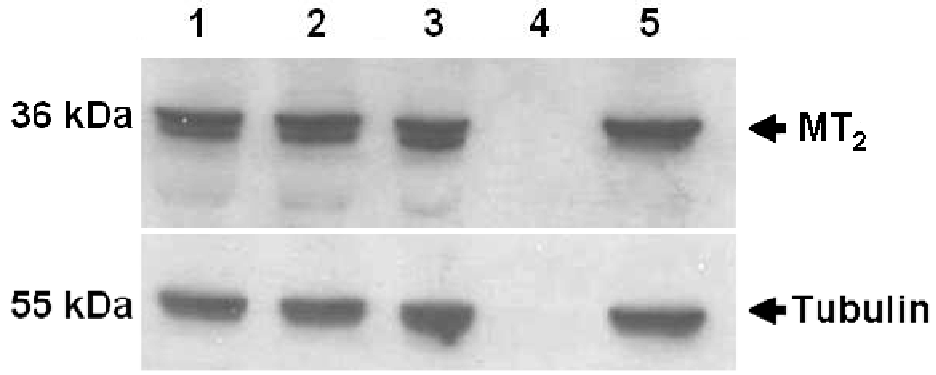


Figure 3. Representative western blot images of the MT_2-receptor protein in porcine coronary artery homogenates. Immunoreactive bands at 36 kDa are for porcine coronary artery samples from 3 different animals (lanes 1-3) and rat heart used as a positive control (lane 5). No immunoreactivity was detected in loading buffer (negative control, lane 4). The figure is representative of images taken from arteries of six different animals (n=6).

2.3.2 Immunohistochemistry

Immunohistochemical analysis confirmed the presence of MT_2-receptors in porcine coronary arteries (Figure 4A). No labeling was observed in the presence of a competitive blocking peptide (Figure 4A, Inset). MT_2-receptors co-localized with α-actin (Figure 4C), thus demonstrating the presence of MT_2-receptors in the coronary arterial smooth muscle cell layer. Specific MT_2-receptor labeling was also observed in the adventitial layer of the coronary arteries.

2.3.3 Pharmacologic studies

Sodium nitroprusside (SNP; 10^{-9} – 10^{-5} M), an exogenous NO-donor (Kowaluk et al., 1992), caused concentration-dependent relaxations in isolated porcine coronary artery rings, without endothelium, contracted with the thromboxane A_2 mimetic, U46619 (1–3 x 10^{-9} M) (Figure 5A). In the presence of melatonin (10^{-7} M), the concentration-response curve to SNP was shifted to the right in a parallel manner (Figure 5A). The pD_2 values for SNP in the absence and presence of melatonin were 7.49 ± 0.1 and 6.79 ± 0.2, respectively ($P < 0.05$). SNP caused complete (i.e. 100%) relaxation in both untreated and melatonin-treated rings. Melatonin itself had no direct effect on resting tension or on the U46619-induced contraction (Yang et al., 2001).

The endothelium-dependent vasodilator, UK14,304 (10^{-9} – 10^{-5} M) (Flavahan et al., 1989; Bockman et al., 1996), caused concentration-dependent relaxations in endothelium-intact coronary arterial rings (Figure 5B). The response to UK14,304 was markedly inhibited in the presence of melatonin (10^{-7} M) (Figure 5B). Melatonin caused a rightward shift in the concentration-response curve to UK14,304 (p$D_{2=}$ 7.65 ± 0.2 vs. 6.73 ± 0.2 in the absence and presence of melatonin respectively; P <0.05), as well as a reduction in the maximal level of relaxation (E_{max} = 80 ± 9% relaxation vs. 53 ± 8% relaxation, in the absence and presence of melatonin, respectively; P <0.05) (Figure 5B).

This effect of melatonin is specific to NO-induced relaxation as melatonin (10^{-7} M) failed to inhibit relaxation induced by isoproterenol (10^{-9}-10^{-5} M), a β-adrenoceptor agonist, in isolated coronary arteries without endothelium (Figure 6). The pD_2 values to isoproterenol in the absence and presence of melatonin (10^{-7} M) were 7.67 ± 0.1 and 7.64 ± 0.1 (P > 0.05), respectively.

In contrast to melatonin, the MT_3-receptor selective agonist, 5-MCA-NAT (10^{-7} M) (Serle et al., 2004), had no effect on the concentration-response curves to either SNP (Figure 7A; P>0.05) or UK 14,304 (Figure 7B; P>0.05). Pretreatment of coronary arterial rings with the selective MT_2-receptor antagonists, 4P-PDOT (10^{-7} M) or luzindole (10^{-7} M) (Dubocovich, 1988; Zlotos, 2005), abolished the inhibitory effect of melatonin on relaxation induced by either SNP (Figure 8A-B) or UK 14,304 (Figure 9A-B). The concentration-response curves to SNP were unaffected by the presence of either antagonist alone (data not shown for the sake of clarity).

2.3.4 Cyclic GMP measurements

SNP (10^{-5} M) significantly increased cyclic GMP levels in coronary arteries (Figure 10). Incubation of coronary arteries with melatonin (10^{-7} M) had no effect on basal cyclic GMP levels but markedly attenuated the SNP-induced increase in cyclic GMP. In the presence of 4P-PDOT (10^{-7} M), the inhibitory effect of melatonin on the SNP-induced increase in cyclic GMP was abolished. 4P-PDOT itself had no effect on cyclic GMP levels.

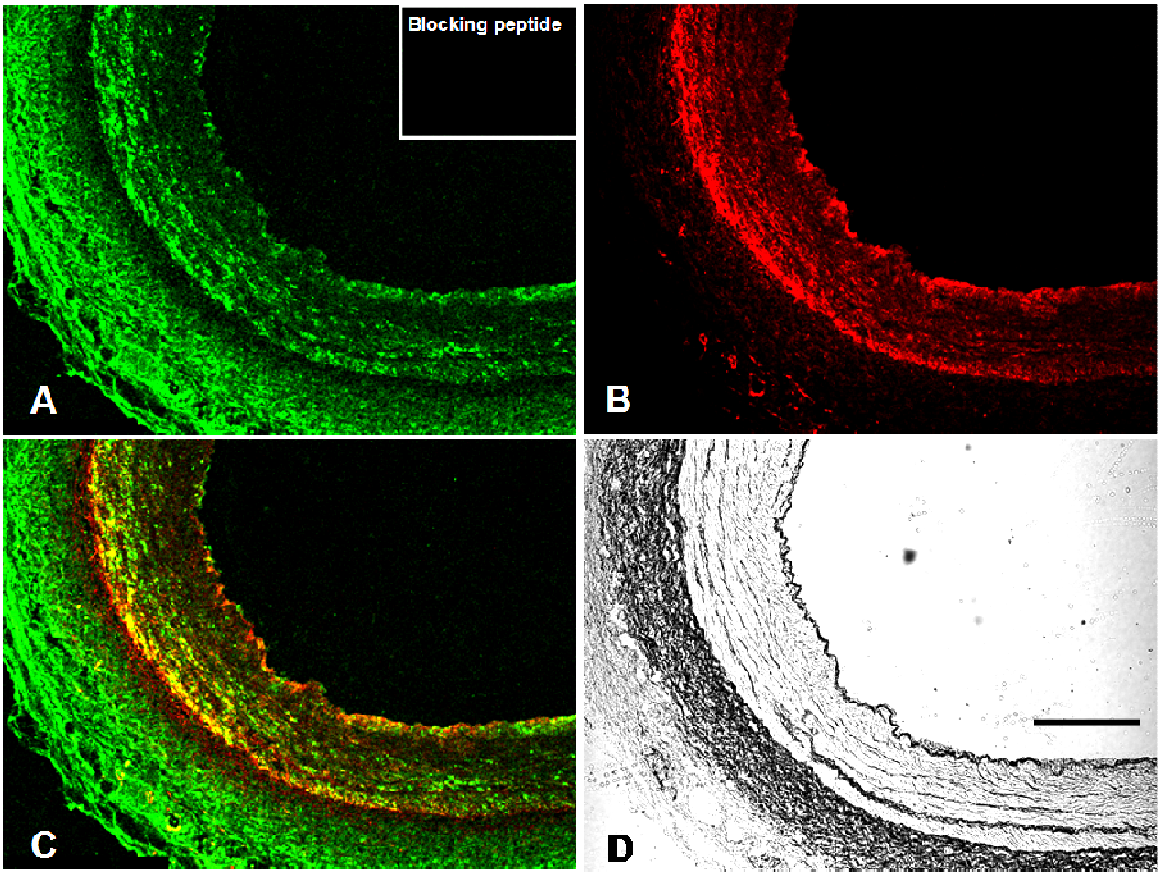


Figure 4. Representative images of immunofluorescent co-localization of MT_2-receptors and smooth muscle cell actin (SMCA) in porcine coronary artery. (A) MT_2-receptor –green fluorescence, (B) SMCA – red fluorescence, (C) co-localized areas showing yellow fluorescence, (D) image taken using light microscopy; insert in (A) demonstrates lack of staining after immunoneutralization of antibody by blocking peptide. Bar = 50 μM. The figure is representative of images taken from arteries of four different animals. (n=4).

2.4 Discussion

The results of the present study demonstrate the presence of structural and functional MT_2-receptors in porcine coronary arterial smooth muscle cells, and that activation of these receptors by melatonin inhibits the ability of the smooth muscle to relax in response to NO. These conclusions are supported by the observations that: 1) MT_2-receptor protein is expressed in porcine coronary arteries; 2) MT_2-receptor proteins co-localized with α-actin, thus indicating that MT_2-receptors are present in the smooth muscle cell layer. MT_2-receptor staining was also present in the adventitial layer; and 3) the inhibitory effect of melatonin on NO-induced smooth muscle

relaxation was abolished in the presence of selective MT_2-receptor antagonists. These findings provide new information regarding the role of MT_2-receptors in regulating vascular function, since prior to the present study the only previously reported vasomotor response to MT_2-receptor activation was vasodilation (Doolen et al., 1998; Masana et al., 2002).

High affinity melatonin binding sites have been identified, characterized, and labeled as MT_1, MT_2 and MT_3 (Dubocovich and Markowska, 2005). Whereas MT_1- and MT_2-receptors belong to the G-protein-coupled family of membrane receptors, the MT_3-receptor is identified as the enzyme quinone reductase 2, an intracellular melatonin binding site (Nosjean et al., 2000). Specific membrane melatonin receptors have been detected in many tissues (e.g. brain, retina, heart, adipocytes) from several different species (Stankov et al., 1992; Mahle et al., 1997; Brydon et al., 2001; Masana et al., 2002; Dubocovich and Markowska, 2005). Within the cardiovascular system, high affinity melatonin binding sites were identified in several vascular beds (Viswanathan et al., 1990; Stankov et al., 1992; Stankov and Fraschini, 1993; Saenz et al., 2002), suggesting a role for melatonin in local control of blood vessel diameter. Indeed, a functional role for melatonin binding sites/receptors was established by studies demonstrating that melatonin causes vasoconstriction in certain vascular beds and vasodilation in others. For example, in rats melatonin causes direct vasoconstriction of cerebral arteries (Capsoni et al., 1995; Geary et al., 1997; Viswanathan et al., 1997) and vasodilation in caudal arteries (Doolen et al., 1998; Masana et al., 2002).

The coronary circulation is also a site of action for the receptor-dependent effects of melatonin. Previous studies from our laboratory demonstrated that, in isolated porcine coronary arteries, melatonin has no direct effect on vasomotor tone but that it inhibits NO-induced relaxation. This effect of melatonin is abolished by the specific MT-receptor antagonist, S20928 (Yang et al., 2001); however, S20928 is nonselective with regard to individual MT-receptor subtypes (Ahn et al., 1992; Petit et al., 1999; Audinot et al., 2003). Thus, in the present study I have used a combination of molecular and pharmacologic techniques to identify the specific MT-receptor subtype mediating the inhibitory effect of melatonin on NO-induced relaxation.

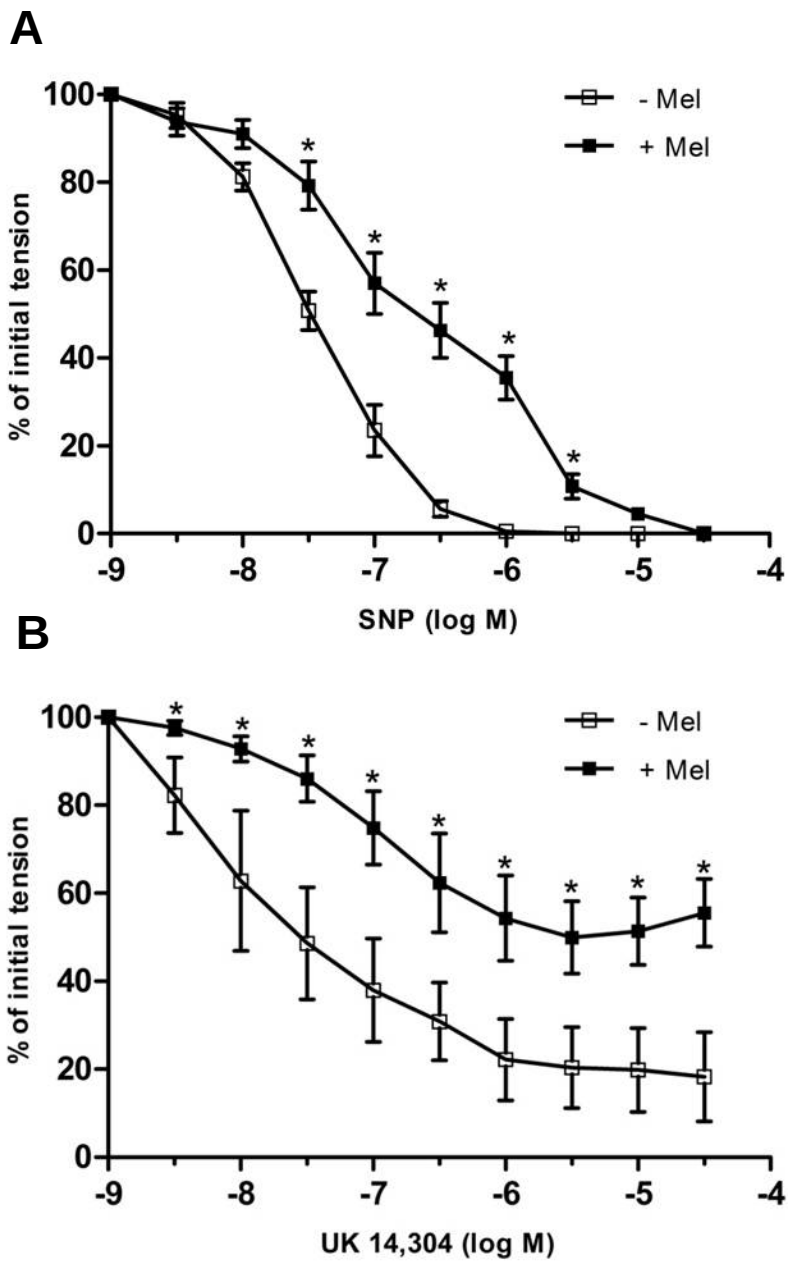


Figure 5. Log concentration-response curves for sodium nitroprusside (SNP, A) or UK14,304 (B) in producing relaxations of isolated porcine coronary arteries (without or with endothelium respectively), in the absence and presence of melatonin (10^{-7} M). Data are expressed as a percentage of the initial increase in tension induced by U46619 ($1–3 \times 10^{-9}$ M), which averaged 2.48 ± 0.2 in control rings and did not differ significantly in rings treated with melatonin. * indicates a statistically significant difference between treatment groups ($P<0.05$; Student's *t*-test). Each point represents the mean ± S.E.M. (n = 4-5).

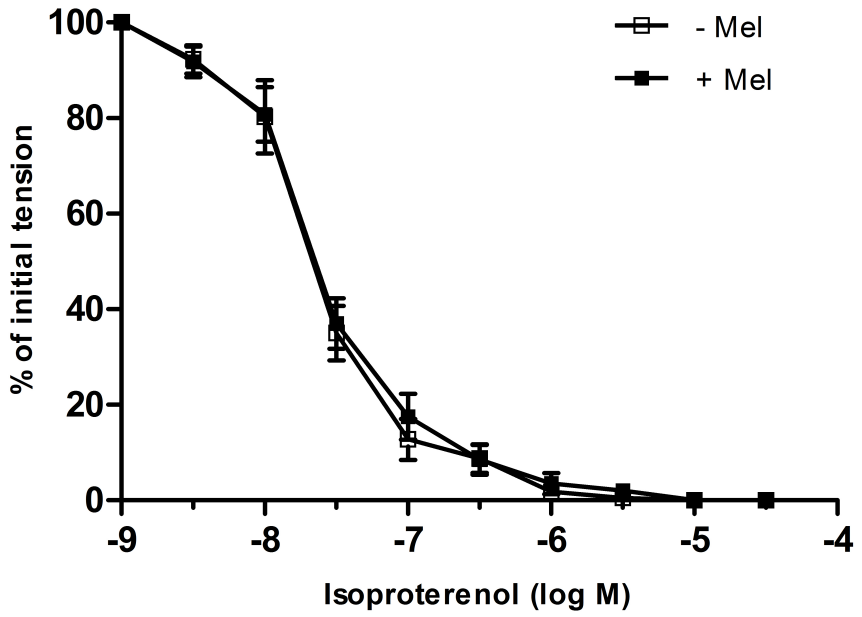


Figure 6. Log concentration-response curves for isoproterenol, a β-adrenoceptor agonist in producing relaxations of isolated porcine coronary arteries (without endothelium) in the absence and presence of melatonin (10^{-7} M). Data are expressed as a percentage of the initial increase in tension induced by U46619 (1–3 × 10^{-9} M), which did not differ significantly between control rings and control rings incubated with melatonin. (*P*>0.05; Student's *t*-test). Each point represents the mean ± S.E.M. (n = 4)

At present, a limited number of potent and selective drugs are available for pharmacologically characterizing MT-receptor subtypes. The most useful agents include luzindole and 4P-PDOT, which at low concentrations (< 0.1μM) are selective antagonists of MT_2-receptors (Boutin et al., 2005), and 5-MCA-NAT, which is a selective MT_3-receptor agonist (Pintor et al., 2001). There are currently no commercially available antagonists that are selective for MT_1-receptors. Using these pharmacologic probes in functional studies, I found that, in contrast to melatonin, 5-MCA-NAT had no effect on NO-induced relaxation of isolated coronary arteries (Figure 7A-B), suggesting that the inhibitory effect of melatonin does not involve activation of MT_3-receptors. The inhibitory effect of melatonin on NO-induced relaxation seems to be specific as melatonin fail to inhibit the relaxation induced by isoproterenol, a β-adrenergic receptor agonist (Figure 6).

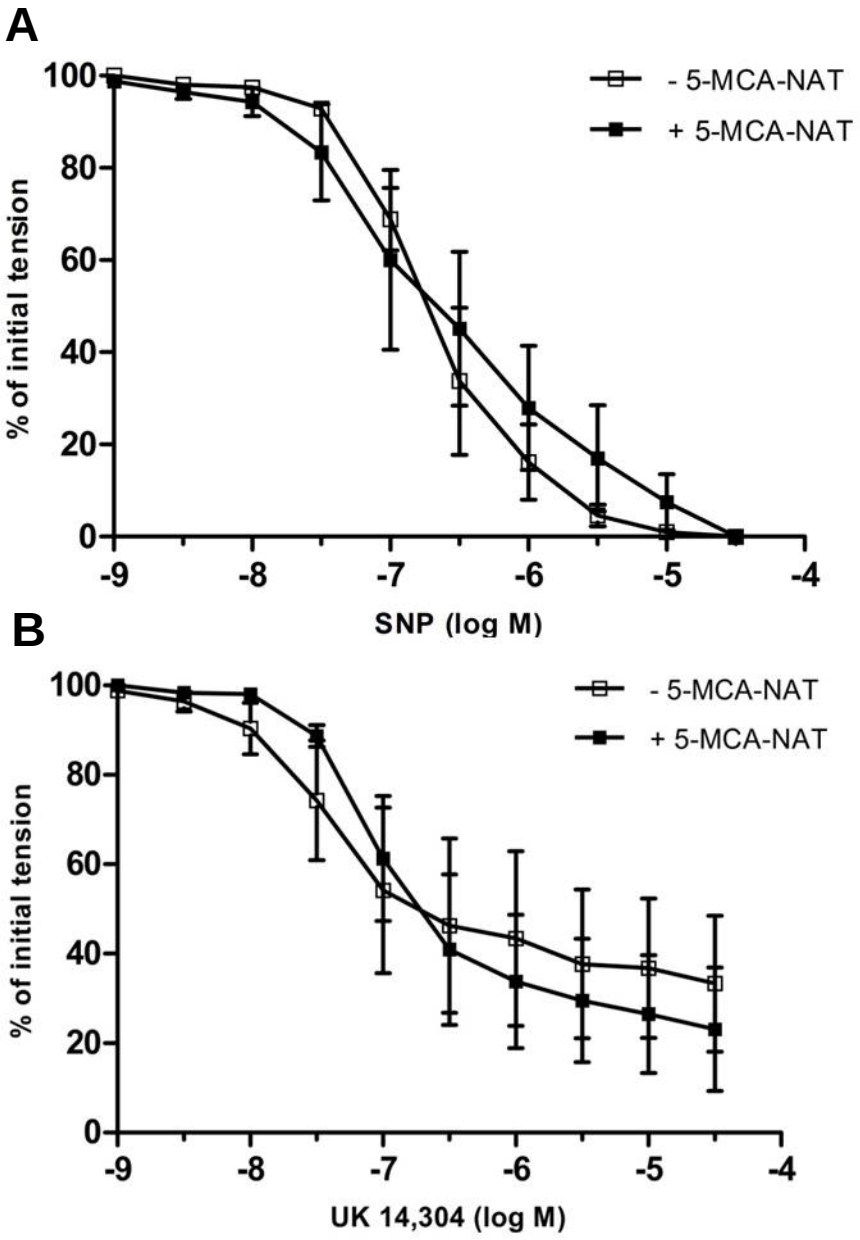


Figure 7. Log concentration-response curves for sodium nitroprusside (SNP, A) or UK14,304 (B) in producing relaxations of isolated porcine coronary arteries (without or with endothelium respectively) in the absence and presence of the 5-MCA-NAT (10^{-7} M). Data are expressed as a percentage of the initial increase in tension induced by U46619 (1–3 × 10^{-9} M), which averaged 5.09 ± 0.9 in control rings and did not differ significantly in rings treated with 5-MCA-NAT (P>0.05; Student's t-test). Each point represents the mean ± S.E.M. (n = 4).

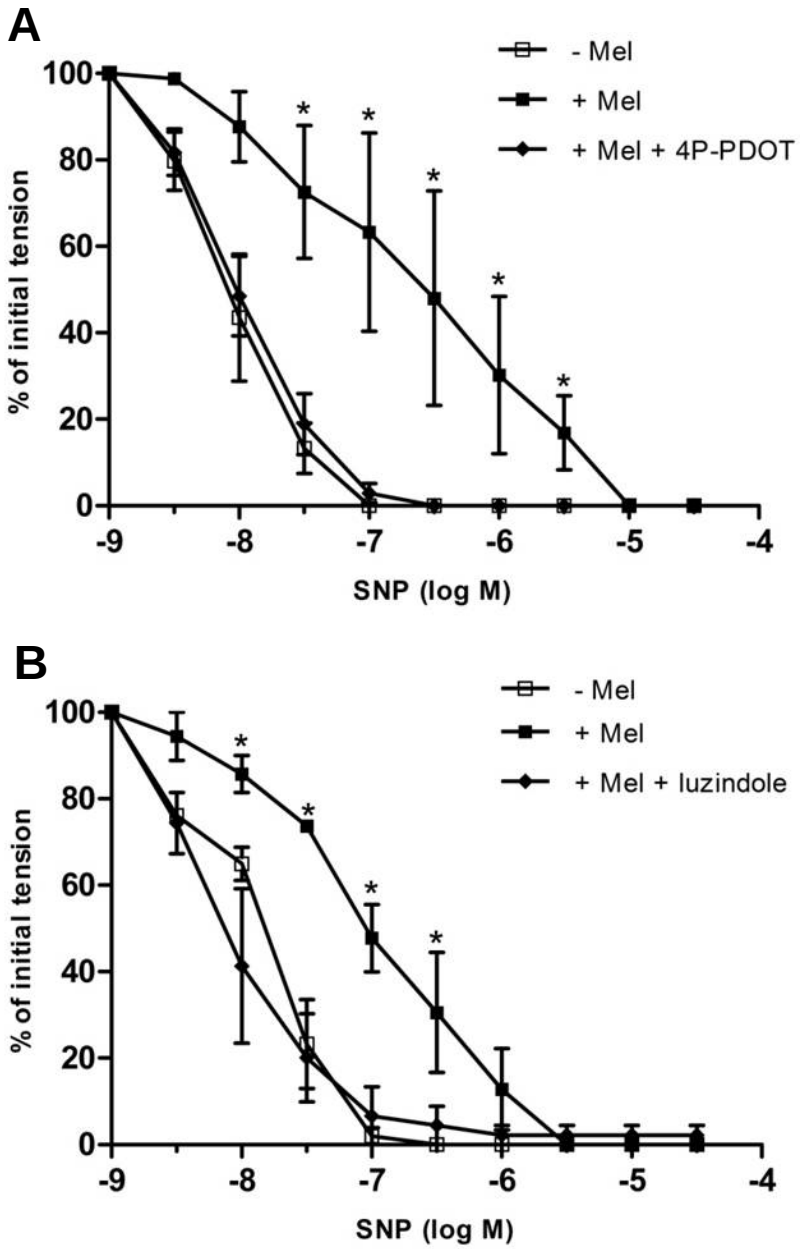


Figure 8. Effect of the selective MT_2-receptor antagonist, 4P-PDOT (10^{-7} M) (A) or luzindole (10^{-7} M) (B) on sodium nitroprusside (SNP)-induced relaxations of isolated porcine coronary arteries (without endothelium) in the absence and presence of melatonin (10^{-7} M). Data are expressed as a percentage of the initial increase in tension induced by U46619 (1–3 x10^{-9} M), which averaged 2.66 ± 0.2 g in control rings and did not differ significantly in rings treated with melatonin, 4P-PDOT, or luzindole. * indicates a statistically significant difference between treatment groups (*P* >0.05; ANOVA). Each point represents the mean ± S.E.M. (n = 4-5).

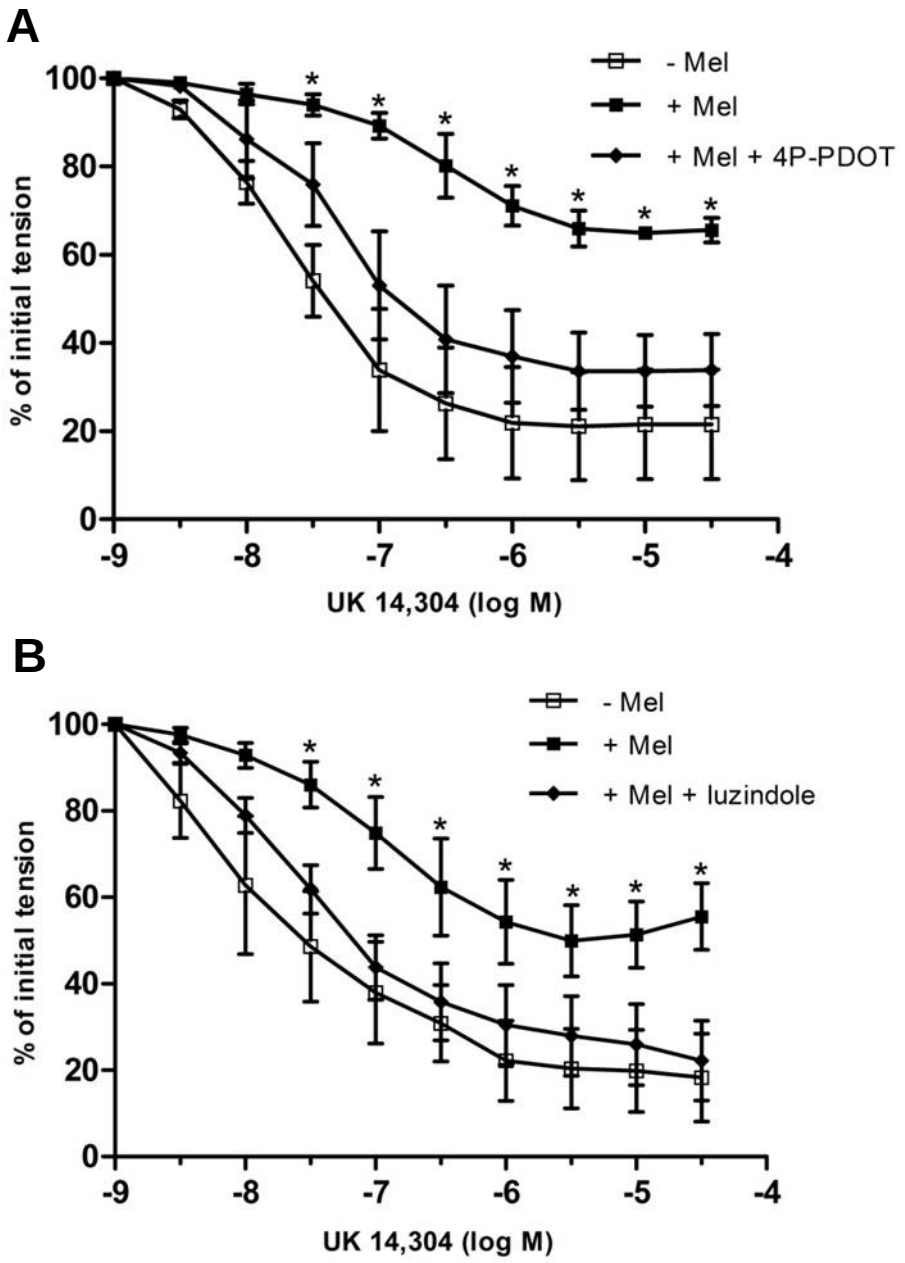


Figure 9. Effect of the selective MT_2-receptor antagonists, 4P-PDOT (10^{-7} M) (A) or luzindole (10^{-7} M) (B) on UK14,304-induced relaxation of isolated porcine coronary arteries (with endothelium) in the absence and presence of melatonin (10^{-7} M). Data are expressed as a percentage of the initial increase in tension induced by U46619 (1–3 x10^{-9} M), which averaged 2.28 ± 0.3 g in control rings and did not differ significantly in rings treated with melatonin, 4P-PDOT, or luzindole. * indicates a statistically significant difference between treatment groups (*P* >0.05; ANOVA). Each point represents the mean ± S.E.M. (n = 4-7).

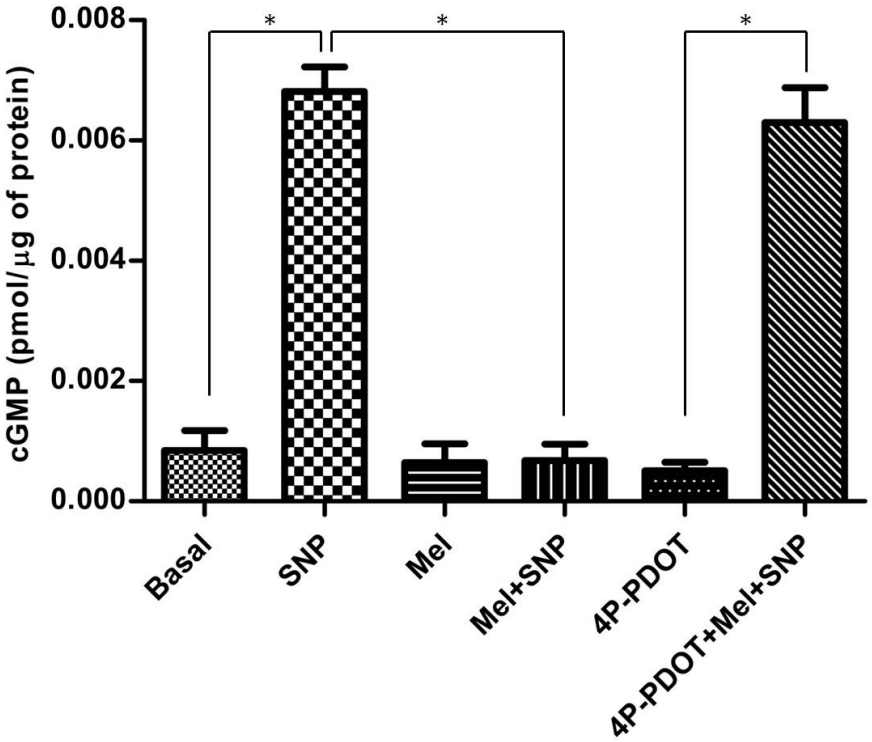


Figure 10. Effect of melatonin (Mel) (10^{-7} M) on sodium nitroprusside (SNP) (10^{-5} M)-induced increases in coronary artery (with endothelium) cyclic GMP levels, in the absence and presence of the MT_2-selective antagonist 4P-PDOT (10^{-7} M). * indicates a statistically significant difference between treatment groups (*P*<0.05; ANOVA). Data are expressed as mean ± S.E.M. (n=6).

However, the inhibitory effect of melatonin on NO-induced relaxation was abolished in the presence of either 4P-PDOT (Figure 8A and 9A) or luzindole, (Figure 8B and 9B) two chemically unrelated antagonists (Boutin et al., 2005), strongly suggesting a role for MT_2-receptors in the response to melatonin in coronary arteries. The expression of MT_2-receptors in the coronary vascular smooth muscle cells, which is the site of action for NO, was confirmed by immunoblot (Figure 3) and immunohistochemical studies (Figure 4). Taken together, these data strongly support the view that melatonin acts on MT_2-receptors in vascular smooth muscle to inhibit the actions of NO in porcine coronary arteries.

The physiologic role of MT_2-receptors in the vasculature is not yet fully understood. In the rat caudal artery, MT_2-receptors are expressed in the vascular smooth muscle layer, where they mediate vasodilation in response to melatonin (Doolen et al., 1998; Masana et al., 2002). MT_2-

receptors are also expressed in human coronary arteries (Ekmekcioglu et al., 2003), but the functional effects of melatonin in the human coronary circulation are unknown. The present findings demonstrate that in addition to mediating vasodilation in some arteries (Doolen et al., 1998; Masana et al., 2002), MT_2-receptors may also be functionally coupled to impaired NO-signaling in others, e.g. coronary arteries, and may provide new strategies for assessing melatonin receptor function in human coronary arteries.

In the present studies, melatonin inhibited porcine coronary arterial relaxation in response to the α_2-adrenoceptor agonist, UK14,304 (Figure 5B), an endothelium-dependent vasodilator that acts solely by releasing NO from endothelial cells in this tissue (Flavahan et al., 1989; Bockman et al., 1996), and by SNP (Figure 5A), an exogenous NO-donor (Kowaluk et al., 1992). One potential site of action for melatonin could be endothelial NO synthase (eNOS). Indeed, melatonin inhibits NO production in rat microvascular endothelial cells (Silva et al., 2007), though this effect is not observed in larger rat arteries (Monroe and Watts, 1998). Although an effect of melatonin on eNOS in porcine coronary arteries cannot be ruled out, the observation that melatonin also inhibits relaxation induced by SNP, an exogenous NO donor that is independent of eNOS (Kowaluk et al., 1992), suggests a site of action for melatonin other than, or in addition to, eNOS. A likely possibility is that melatonin acts directly on the vascular smooth muscle cells, which express MT_2-receptors (Figures 3 and 4) and are the primary site of action for the vasorelaxing effect of NO. The primary mechanism by which NO relaxes vascular smooth muscle is by increasing intracellular cyclic GMP levels, followed by activation of protein kinase G and the subsequent phosphorylation of several regulatory proteins (Ignarro et al., 1981; Murad, 1986). Thus, my observation that melatonin attenuates the NO-induced increase in intracellular cyclic GMP levels in coronary arteries (Figure 10) provides a plausible mechanism that could account for the inhibitory action of melatonin on relaxation to either endogenous or exogenous NO. Moreover, the effects of melatonin on both NO-induced increases in cyclic GMP and arterial relaxation were attenuated by pharmacologic blockade of MT_2-receptors (Figure 10), further suggesting that MT_2-receptors mediate both effects of melatonin and that they are functionally linked to one another. These results are consistent with

previous reports that activation of melatonin receptors is linked to decreased cyclic GMP accumulation in some other cell types (Petit et al., 1999; Stumpf et al., 2008).

Although the circulating peak plasma melatonin concentration generally ranges from 0.5-1 nM, higher tissue melatonin concentrations exceeding plasma melatonin concentrations may occur due to the high lipophilicity of melatonin and existence of extrapineal sources of melatonin including bone marrow, brain, retina, gastrointestinal tract, skin and particularly relevant to this study, platelets, leukocytes, mast cells and endothelial cells (Bubenik, 2002; Tan et al., 2003; Pandi-Perumal et al., 2008a; Slominski et al., 2008). Indeed melatonin concentrations 2 to 3 orders of magnitude higher than those typically found in blood have been reported in bile and bone marrow (Tan et al., 1999a; Tan et al., 1999b). Moreover, the concentrations of melatonin in the gastrointestinal tract are 10-100x higher than in the plasma and the total amount of melatonin in the gastrointestinal tract is around 400x higher than the amount of melatonin in the pineal gland (Bubenik et al., 2008). In these tissues melatonin may act as an autacoid or paracoid signal thereby influencing local physiology (Pandi-Perumal et al., 2008b; Slominski et al., 2012). This, as well as the therapeutic use of melatonin in treating sleep disorders, further increases the potential for elevations in the local tissue melatonin concentrations. A single oral dose of 4 or 80 mg of melatonin in healthy human subjects results in the peak serum melatonin concentrations of 24.5 nM (DeMuro et al., 2000) or 430.5 nM (Waldhauser et al., 1984), respectively. Thus based on the lipophilicity of melatonin and existence of extrapineal sources of melatonin, it is plausible that local tissue melatonin concentration may reach pharmacological level similar to one used in this study resulting in the inhibition of NO-induced relaxations.

2.5 Conclusion

Taken together, the results of the present study support the view that melatonin acts on MT_2-receptors on vascular smooth muscle cells to inhibit coronary arterial relaxation induced by either exogenous or endogenous NO, thus demonstrating a novel function for MT_2-receptors in the vasculature. Since MT_2-receptors are expressed in human coronary arteries and their expression is

altered in cardiovascular disease (Ekmekcioglu et al., 2003) these findings may provide clues as to the physiological and pathophysiological role of melatonin in the human coronary circulation.

CHAPTER 3. ROLE OF PHOSPHODIESTERASE TYPE-5 IN THE INHIBITORY EFFECT OF MELATONIN ON NO-INDUCED RELAXATIONS OF PORCINE CORONARY ARTERIES

3.1 Introduction

An increasing body of evidence supports a role for melatonin in the local regulation of vascular tone (Krause et al., 2000; Pandi-Perumal et al., 2008b); however, the mechanisms underlying the vasomotor effects of the hormone are not yet clear. Melatonin has both direct and indirect effects on the vasculature and may cause either vasoconstriction or vasodilation depending on the origin of the blood vessel under investigation. For example, melatonin directly contracts rat cerebral arteries in vitro (Capsoni et al., 1995; Geary et al., 1997; Viswanathan et al., 1997), whereas in isolated caudal arteries of the rat melatonin has no direct action, but potentiates contractile responses induced by other vasoconstrictors (Viswanathan et al., 1990; Evans et al., 1992; Krause et al., 1995; Mahle et al., 1997; Ting et al., 1997; Geary et al., 1998). Conversely, melatonin dilates rat and rabbit aorta, iliac, renal and basilar arteries (Shibata et al., 1989; Weekley, 1991). In humans, melatonin increases blood flow in certain vascular beds (e.g. forearm) while decreasing flow in others (e.g. renal) (Cook et al., 2011). The vasomotor effects of melatonin are mediated primarily via activation of two distinct receptor subtypes, termed MT_1 and MT_2 (Dubocovich et al., 2003), that are present in the vasculature. Thus, the heterogeneity in responses to melatonin may be dependent, in part, on the relative distribution and function of specific melatonin receptor subtypes in individual blood vessels.

MT_2-receptors are present in human coronary arteries, and although their function is unknown, MT_2-receptor expression is altered in patients with coronary artery disease (Ekmekcioglu et al., 2003). In porcine coronary arteries, melatonin impairs vascular smooth muscle relaxation in response to nitric oxide (NO) (Yang et al., 2001; Tunstall et al., 2011). This inhibitory effect of melatonin on NO-induced vasorelaxations is mediated by activation of MT_2-melatonerigic receptors, which are expressed in coronary vascular smooth muscle cells (Tunstall et al., 2011).

Since it is widely held that NO-induced relaxation of vascular smooth muscle results from increased intracellular accumulation of cyclic GMP, which in turn activates downstream signaling events that lead to relaxation (Ignarro and Kadowitz, 1985; Ignarro, 1989; Lincoln and Cornwell, 1991), interference with the NO/cyclic GMP signaling cascade may provide a link by which MT_2-receptor activation is coupled to impaired NO-induced responses in coronary arteries.

Intracellular levels of cyclic GMP are tightly regulated by the opposing activities of guanylyl cyclase and cyclic GMP specific phosphodiesterase (PDE) (Wyatt et al., 1998). More than 10 different PDE isoforms have been identified, including PDE5 which is the major cyclic GMP-hydrolyzing enzyme expressed in most smooth muscle cells (Bender and Beavo, 2006). PDE5 is highly specific for cyclic GMP and plays a pivotal role in NO/cyclic GMP signaling in vascular smooth muscle cells (Mullershausen et al., 2001; Bender and Beavo, 2006; Murthy, 2008). A net decrease in intracellular cyclic GMP levels may result from increased degradation by PDE (Wyatt et al., 1998); thus, increased PDE5 activity represents a potential mechanism by which melatonin could inhibit responses to NO. At present, the role of PDE5, if any, in the modulation of cyclic GMP levels by melatonin in vascular smooth muscle is not known. Therefore, I tested the hypothesis that melatonin activates PDE5 in coronary arteries, thereby decreasing intracellular cyclic GMP accumulation in response to NO and inhibiting NO-induced relaxation.

3.2 Materials and methods

3.2.1 Materials

The following drugs were used: bradykinin, melatonin, sodium nitroprusside (Sigma Chemical Co., St Louis, MO, USA); 4P-PDOT (4-Phenyl-2-propionamidotetralin), 8-Br-cGMP, zaprinast (Tocris, Ellisville, MO, USA); and U46619 (Cayman Chemical Company, Ann Arbor, MI, USA). Drug solutions were prepared daily, kept on ice, and protected from light until used. All drugs were dissolved initially in distilled water with the exception of melatonin and 4P-PDOT, which were dissolved in ethanol, and zaprinast, which was dissolved in DMSO, before further dilution in distilled water. Drugs were added to the organ chambers in volumes not greater than 0.2 ml (i.e. 0.8 % of the total volume). Drug concentrations are reported as final molar concentration in the

organ chamber. The composition of the physiological salt solution was as follows (in mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1 (pH=7.4).

3.2.2 Methods

3.2.2.1 Tissue preparation

Fresh porcine hearts were obtained from a local abbatoir and were immediately immersed in cold physiological salt solution. After transfer to the laboratory, the left anterior descending coronary artery was dissected free from surrounding myocardium, cleaned of adherent fat and connective tissue, and cut into rings 4–5 mm in length. The endothelium was removed by gently rubbing the intimal surface with a fine forceps. Four to eight coronary arterial rings were prepared from each heart. In some rings, the endothelium was removed by gently rubbing the intimal surface with a fine forceps.

3.2.2.2 Pharmacologic studies

Coronary arterial rings were suspended in water-jacketed organ chambers filled with 25 ml of physiological salt solution, as previously described (Yang et al., 2001). The organ chamber solution was aerated with a mixture of 95% O_2/5% CO_2 and the temperature was maintained at 37˚C throughout the experiment. Each ring was suspended by means of two fine stainless-steel wire clips passed through the lumen; one clip was anchored inside the organ chamber, the other connected to a force transducer (Model FT03, Grass Instrument Company, Quincy, MA, USA). Isometric tension was measured and recorded on a Grass polygraph. The tissues were stretched progressively to the optimal point of their length–tension relationship, using KCl (20 mM) to generate a standard contractile response. After this procedure, the preparations were allowed to equilibrate at their optimal length for at least 30 min prior to further exposure to any vasoactive substances. Removal of the endothelium was confirmed in each preparation by the absence of relaxation to the endothelium-dependent vasodilator, bradykinin (10^{-7} M).

Relaxation of coronary arteries was studied in rings contracted with 9, 11-dideoxy-11α,9α-epoxymethano-$PGF_{2\alpha}$ (U46619, 3 x 10^{-9} M), a thromboxane A_2-mimetic. After the U46619-induced

contraction had reached a stable plateau, relaxation responses to increasing concentrations of sodium nitroprusside (SNP; 10^{-9}–10^{-5} M) or 8-Br-cGMP (10^{-9}–10^{-4} M) were obtained in the presence or absence of melatonin (10^{-7} M), which was added to the organ chamber immediately prior to the addition of U46619. In some experiments, the preparations were incubated with either the cyclic GMP specific phosphodiesterase inhibitor, zaprinast (10^{-5} M) or sildenafil (10^{-7} M), or the MT_2-receptor antagonist, 4P-PDOT (10^{-7} M), for 30 min prior to exposure to melatonin. These inhibitors remained in contact with the tissues throughout the remainder of the experiment.

3.2.2.3 Cyclic GMP measurements

An enzyme linked immunosorbent assay was used to measure cyclic GMP levels in porcine coronary arteries. Coronary arterial rings were suspended in water-jacketed organ chambers filled with 25 ml of physiological salt solution and were allowed to equilibrate for at least 1 hr at 37˚C. After the equilibration period, coronary artery rings were treated with SNP (5 min, 10^{-5} M) in the absence or presence of melatonin (5 min, 10^{-7} M). In some experiments, rings were pretreated for 20 min with zaprinast (10^{-5} M) or 4P-PDOT (10^{-7} M) before exposing the rings to melatonin and SNP. After drug treatments, rings were frozen in liquid nitrogen and homogenized with an IKA® Ultra, Turrax-T8 homogenizer (IKA works. Inc., Wilmington, NC, USA) at 4˚C in 0.1 N hydrochloric acid. The tissue homogenates were centrifuged for 10 min at ≥ 600g. The cyclic GMP and total protein content were determined in the supernatant as per the direct cyclic GMP enzyme immunoassay kit (Assay design, Ann Arbor, MI, USA) and a Bio-Rad D_c protein assay kit (Biorad Laboratories, Hercules, CA, USA), respectively. The cyclic GMP levels were expressed as pmol/µg of protein.

3.2.2.4 Detection of PKG1, PDE5/Phospho-PDE5 by immunoblot

Coronary arterial rings were incubated for 24 hrs in serum-free DMEM (supplemented with 100U/ml penicillin and 100 mg/ml streptomycin; 37 ± 0.5 ˚C, 20% O_2–5% CO_2, pH 7.4) in the presence of solvent (DMSO or DDW) or SNP (10^{-5} M). In some experiments, tissues were first incubated with 1H-(1,2,4) oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) (10^{-5} M), an inhibitor of soluble guanylyl cyclase, before exposing to SNP (10^{-5} M). In a separate series of experiments, coronary

arterial rings were pretreated with the PKG1-inhibitors Rp-8-Br-PET-cGMPS ($3x10^{-7}$ M) or DT-2 (10^{-5} M) for 30 minutes in organ chambers before exposing to melatonin (5 min, 10^{-7} M). After drug treatments, rings were frozen in liquid nitrogen, and immunoblotting was performed using anti-PKG1, PDE5 or phosphor-PDE5 antibody as described below.

Following incubation with SNP (10^{-5} M) for 24 hrs, coronary arterial rings were repeatedly rinsed and suspended in water-jacketed organ chambers filled with 25 ml of physiological salt solution and were allowed to equilibrate for at least 1 hr at 37˚C. In a separate series of experiments, coronary arterial rings were suspended for equilibration without being previously exposed to either SNP (10^{-5} M) for 24 hrs. After equilibration period coronary arterial rings were treated with melatonin (5 min, 10^{-7} M).

After drug treatments, rings were frozen in liquid nitrogen and homogenized with an IKA® Ultra, Turrax-T8 homogenizer (IKA works. Inc., Wilmington, NC, USA) at 4˚C in lysis buffer, supplemented with a protease and phosphatase inhibitor cocktail (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The tissue homogenates were kept on ice for 10 min and afterwards centrifuged for 10 min at 10,000g. Supernatants were collected for protein determination and Western blotting. Protein concentration was assayed using a Bio-Rad D_c Protein assay kit (Biorad Laboratories, USA). Aliquots of supernatants containing equal amounts of protein (60-100 µg) were separated on 7.5 % polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins were electrophoretically transferred onto a PVDF membrane (Biorad Laboratories, USA). Blots were blocked for 30-40 minutes with 5% nonfat dry milk in phosphate buffered saline (PBS, pH 7.4) and then incubated overnight at 4˚C with a primary antibody specific for PKG1 (Santa Cruz Biotechnology Inc., USA) or PDE5 or Phospho-PDE5 (FabGennix International Inc., Frisco, TX, USA) using a dilution of 1:1000, 1:1000 or 1:100-200 respectively. Membranes were washed two times for 15 minutes using PBS Tween-20 (PBST) and incubated with a horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology Inc., USA). To ensure equal loading, the blots were analyzed for β-tubulin expression using an anti-β-tubulin antibody (Santa Cruz Biotechnology). Immunodetection was performed using an enhanced

chemiluminescence light detecting kit (Thermo Scientific, Rockford, IL, USA). In preliminary control experiments, no immunoreactive bands were observed in samples treated with loading buffer alone or with specific blocking peptides for the primary antibodies (provided by the manufacturer). The images are representative of six different samples obtained from six different animals.

3.2.2.5 Data analysis

Relaxation responses are expressed as a percentage of the initial tension induced by U46619. For each vasodilator, both the maximal percent relaxation (E_{max}) and the concentration necessary to produce 50% of its own maximal response (EC_{50}) were determined. The EC_{50} values were converted to the negative logarithms and expressed as -log molar EC_{50} (pD_2). Results are expressed as mean ± S.E. and *n* refers to the number of animals from which blood vessels were taken. Immunoblots were analyzed to determine the density of the individual protein band and normalized with respect to the density of the corresponding PDE5 or β-tubulin protein band. Values were compared by Student's *t*-test for paired or unpaired observations, or by analysis of variance and a post hoc Bonferroni's multiple comparison analysis to determine significance between groups, as appropriate. Values were considered to be significantly different when $P < 0.05$.

3.3 Results

3.3.1 Pharmacologic studies

Sodium nitroprusside (SNP; 10^{-9} – 10^{-5} M), an NO-donor (Kowaluk et al., 1992) (Figure 11A), and 8-Br-cyclic GMP (8-Br-cGMP; 10^{-6} – 10^{-4} M), a stable, cell permeable analog of cyclic GMP (Corbin et al., 1986; Francis et al., 1988) (Figure 11B)**,** each caused concentration-dependent relaxations in isolated porcine coronary artery rings contracted with the thromboxane A_2 mimetic, U46619 (Figure 11A-B). In the presence of melatonin (10^{-7} M), the concentration-response curve to SNP (Figure 11A), but not 8-Br-cGMP (Figure 11B), was shifted to the right in a parallel manner. The pD_2 values for SNP in the absence and presence of melatonin were 7.45 ± 0.1 and 6.54 ± 0.1, respectively ($P < 0.05$). SNP and 8-Br-cGMP each caused complete (i.e. 100%) relaxation in both

untreated and melatonin-treated rings (Figure 11A-B). Melatonin itself had no direct effect on resting tension or on the U46619-induced contraction, as reported previously (Yang et al., 2001).

Incubation of coronary arterial rings with the selective MT_2-receptor antagonist, 4P-PDOT (10^{-7} M) (Dubocovich et al., 1997; Browning et al., 2000), had no effect on the concentration-response curve to SNP (data not shown for the sake of clarity) but abolished the inhibitory effect of melatonin on SNP-induced relaxation (Figure 8A). Moreover, in the presence of the cyclic GMP specific PDE5 inhibitor, zaprinast (10^{-5} M) or sildenafil (10^{-7} M) (Mullershausen et al., 2001; Bender and Beavo, 2006), melatonin had no effect on the SNP concentration-response curve (Figure 12A-B). The contractile response to U46619 was unaffected by the presence of 4P-PDOT, zaprinast or sildenafil (see Figure 8 & 12A-B legends).

3.3.2 Cyclic GMP measurements

SNP produced a significant increase in intracellular cyclic GMP levels in coronary arteries (Figure 13). Melatonin had no effect on basal cyclic GMP levels but markedly inhibited the SNP-induced increase in intracellular cyclic GMP (Figure 13). Similar to the results obtained in the vasorelaxations studies described above, the effect of melatonin on SNP-induced increases in intracellular cyclic GMP levels was abolished in the presence of either the selective PDE5 inhibitor, zaprinast (10^{-5} M), or the MT_2-receptor antagonist, 4P-PDOT (10^{-7} M) (Figure 10 and 13).

3.3.3 Immnoblot detection of PDE5/Phospho PDE5

The expression of PDE5 protein in porcine coronary arteries was determined by immunoblot analysis. A strong immunoreactive band at 95 kDa corresponding to PDE5 (Moreno et al., 2004; Ni et al., 2004) was detected in immunoblots of coronary artery homogenates (Figure 14A). Immunoblot analysis of coronary arteries exposed to melatonin (10^{-7} M) demonstrated a significant increase in PDE5 phosphorylation as compared to untreated controls (Figure 14B, C). The MT_2-receptor antagonist, 4P-PDOT (10^{-7} M), had no effect on basal phospho-PDE5 levels, but completely abolished the stimulatory effect of melatonin (10^{-7} M) on PDE5 phosphorylation (Figure 14B-C).

3.3.4 PKG1 down regulation and pharmacologic blockade of PKG1

Incubation of porcine coronary artery rings with SNP (10^{-5} M; 37 ºC in serum free DMEM x 24 hr) caused a significant decrease in PKG1 protein expression (Figure 15A-B). The effect of SNP on PKG1 expression was abolished by ODQ (10^{-5} M), a selective soluble guanylyl cyclase inhibitor (Garthwaite et al., 1995) (Figure 15A-B). In control rings not subjected to PKG1 downregulation, melatonin (10^{-7} M) caused a significant increase in PDE5 phosphorylation (Figure 14 B-C and 16A-B). The effect of melatonin on PDE5 phosphorylation was markedly attenuated in those arteries that were first treated with SNP (10^{-5} M; 24 hrs) to induce down regulation of PKG1 (Figure 16A-B). Similarly, pharmacologic blocked of PKG1 with the PKG1-inhibitors Rp-8-Br-PET-cGMPS ($3x10^{-5}$ M) or DT-2 (10^{-5} M) significantly attenuated ($p<0.05$) the melatonin (10^{-7} M) induced increase in PDE5 phosphorylation in porcine coronary arteries (Figure 17).

3.4 Discussion

The major finding of the present study is that the cyclic GMP-hydrolyzing enzyme, PDE5, plays a key role in the inhibitory effect of melatonin on NO-induced relaxation of coronary arteries. This conclusion is supported by the observations that: 1) melatonin stimulates the phosphorylation of PDE5 in coronary arteries, which markedly enhances the catalytic activity of the enzyme and thereby increases the degradation of intracellular cyclic GMP (Corbin et al., 2000; Mullershausen et al., 2001; Rybalkin et al., 2002); 2) the inhibitory effect of melatonin on NO-induced relaxation and increased intracellular cyclic GMP accumulation is abolished in the presence of zaprinast or sildenafil, the selective PDE5 inhibitor (Mullershausen et al., 2001; Bender and Beavo, 2006); and 3) the effects of melatonin on PDE5 phosphorylation, cyclic GMP accumulation, and vasorelaxation are all blocked by the selective MT_2-receptor antagonist, 4P-PDOT. The results further suggest that melatonin-induced phosphorylation of PDE5 may be mediated, in part, by activation of PKG1 since downregulation of the kinase significantly attenuates melatonin-induced PDE5 phosphorylation. These findings provide a novel mechanistic basis for the inhibitory action of melatonin on NO-induced vasorelaxation and provide new insight into potential mechanisms by which melatonin may regulate blood vessel diameter.

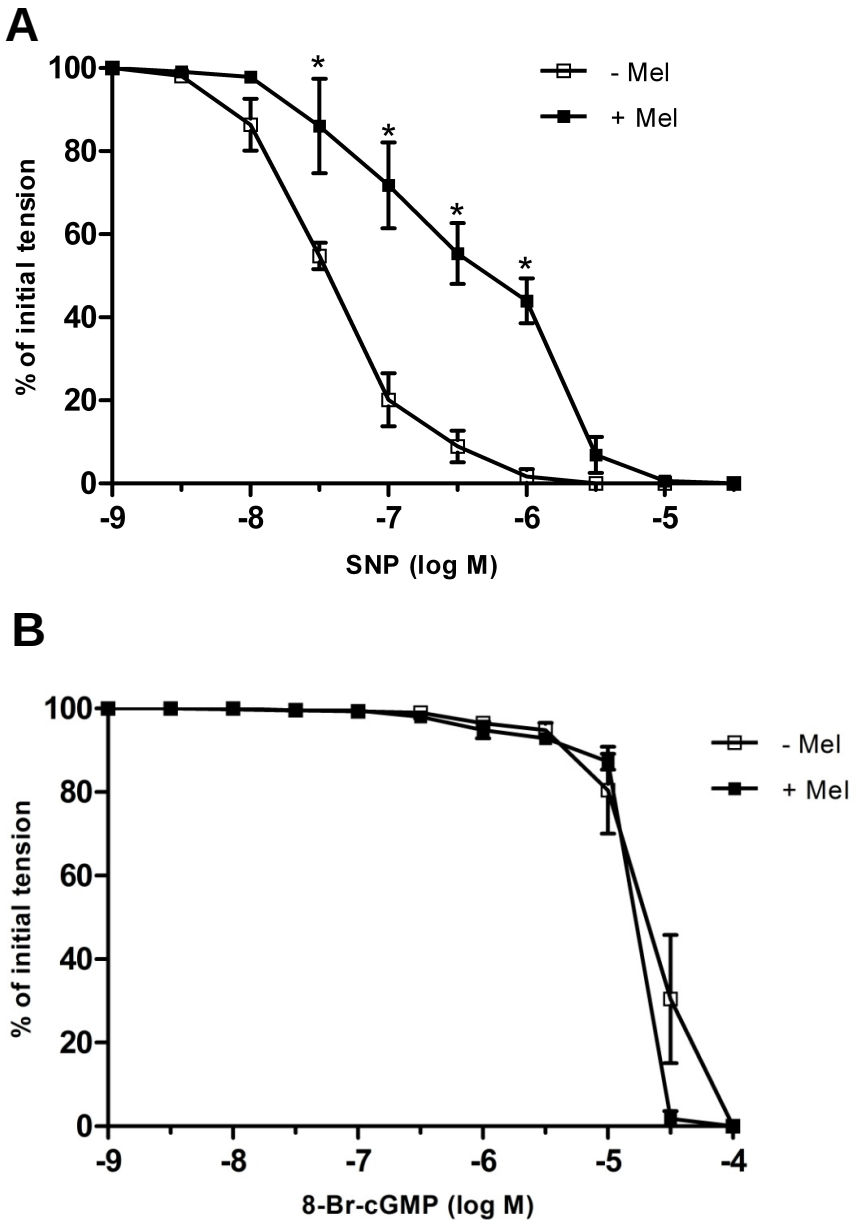


Figure 11. Log concentration-response curves for sodium nitroprusside (A) or 8-Bromo-cGMP (B) in producing relaxations of isolated porcine coronary arteries (without endothelium) in the absence and presence of melatonin (10^{-7} M). The data are expressed as a percentage of the U46619 (3×10^{-9} M)-induced increase in tension, which averaged 5.61 ± 0.6 g in control rings and did not differ significantly in rings incubated with melatonin. * indicates a statistically significant difference in the presence of melatonin ($P<0.05$; Student *t*-test). Each point represents the mean ± S.E.M. (n = 5-8).

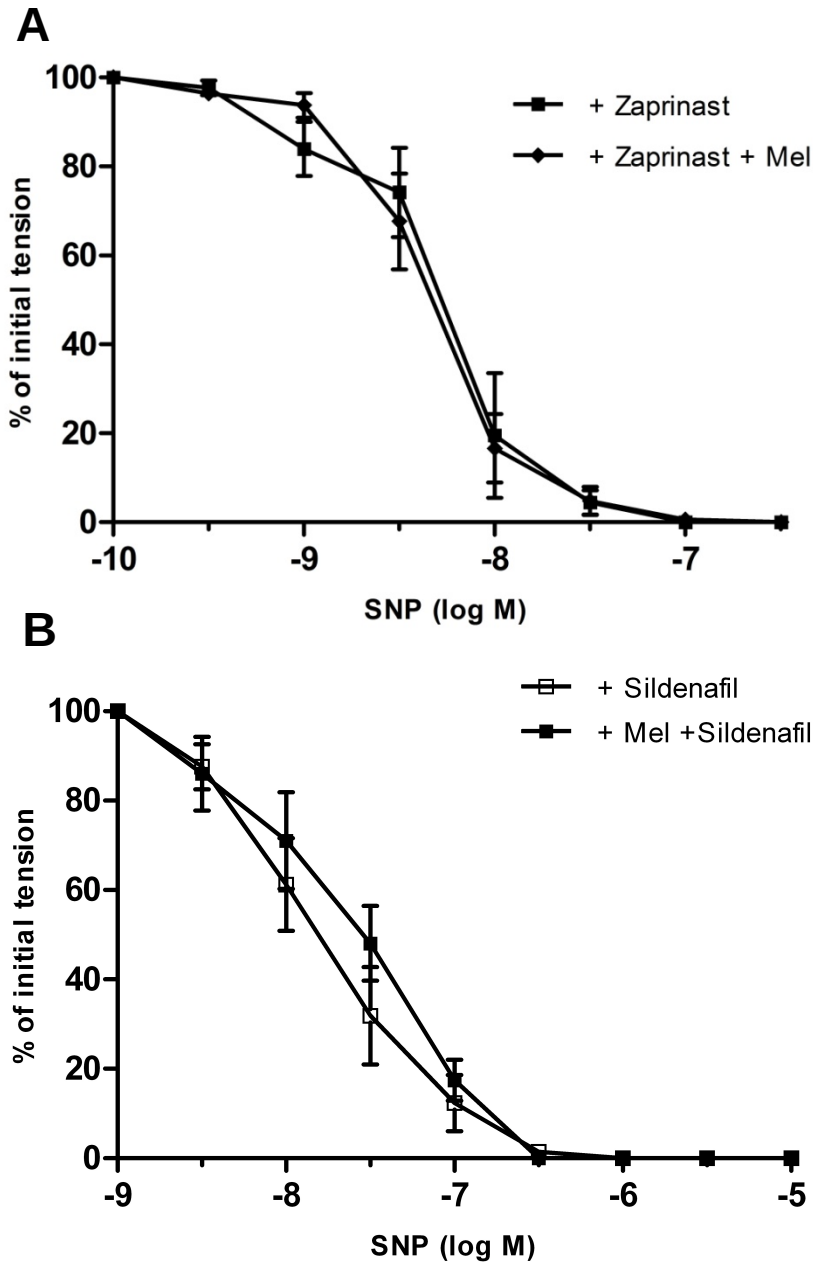


Figure 12. A: Effect of melatonin (10^{-7} M) on SNP-induced relaxation of isolated porcine coronary arteries (without endothelium) in the presence of the cyclic GMP specific PDE5 inhibitor, zaprinast (10^{-5} M) (A) or sildenafil (10^{-7} M) (B). The data are expressed as a percentage of the U46619 (3×10^{-9} M)-induced increase in tension, which averaged 6.44 ± 1.0 g in control rings and did not differ significantly in rings incubated with either zaprinast or sildenafil. (P<0.05; Student t-test). Each point represents the mean ± S.E.M. (n = 6-8).

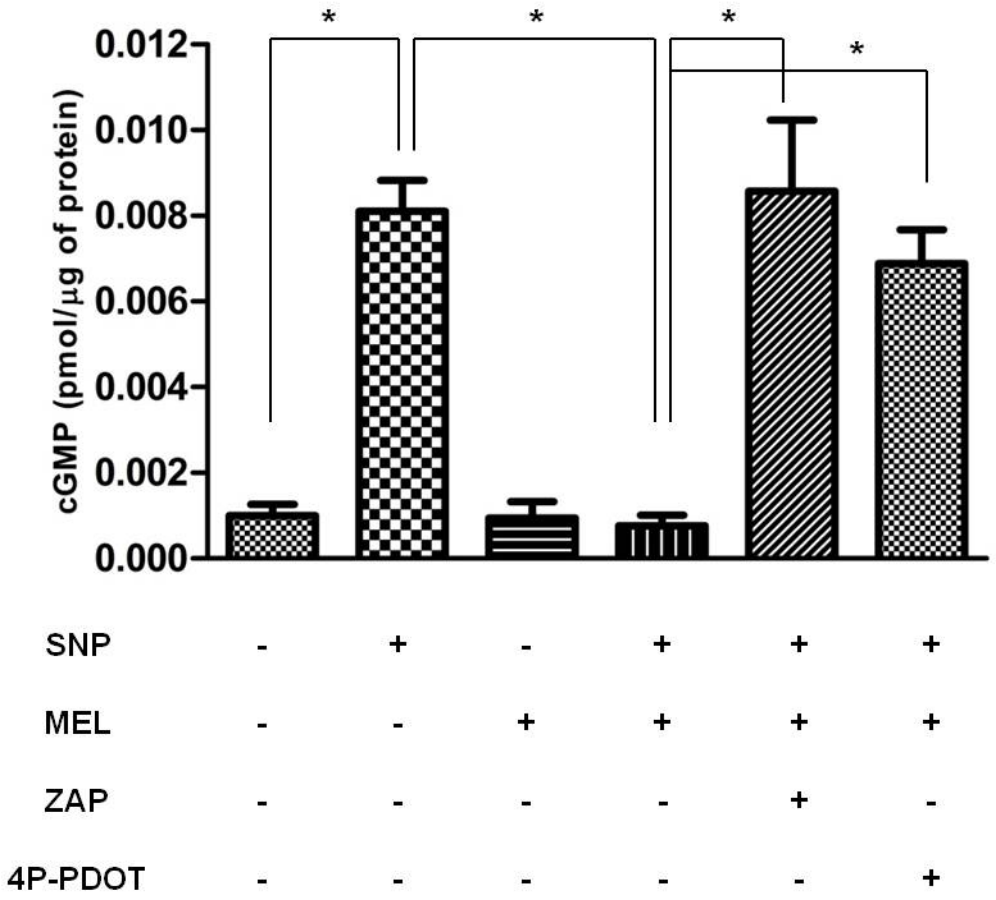


Figure 13. Effect of the cyclic GMP specific PDE5 inhibitor zaprinast (10^{-5} M), and the selective MT_2-receptor antagonist, 4P-PDOT (10^{-7} M), on the SNP (10^{-5} M)-induced increase in cyclic GMP levels in porcine coronary arteries (with endothelium) in the presence and absence of melatonin (10^{-7} M). * indicates a statistically significant difference between groups (P<0.05; ANOVA). The cyclic GMP levels are expressed as pmol/µg of protein.. (n= 6-7).

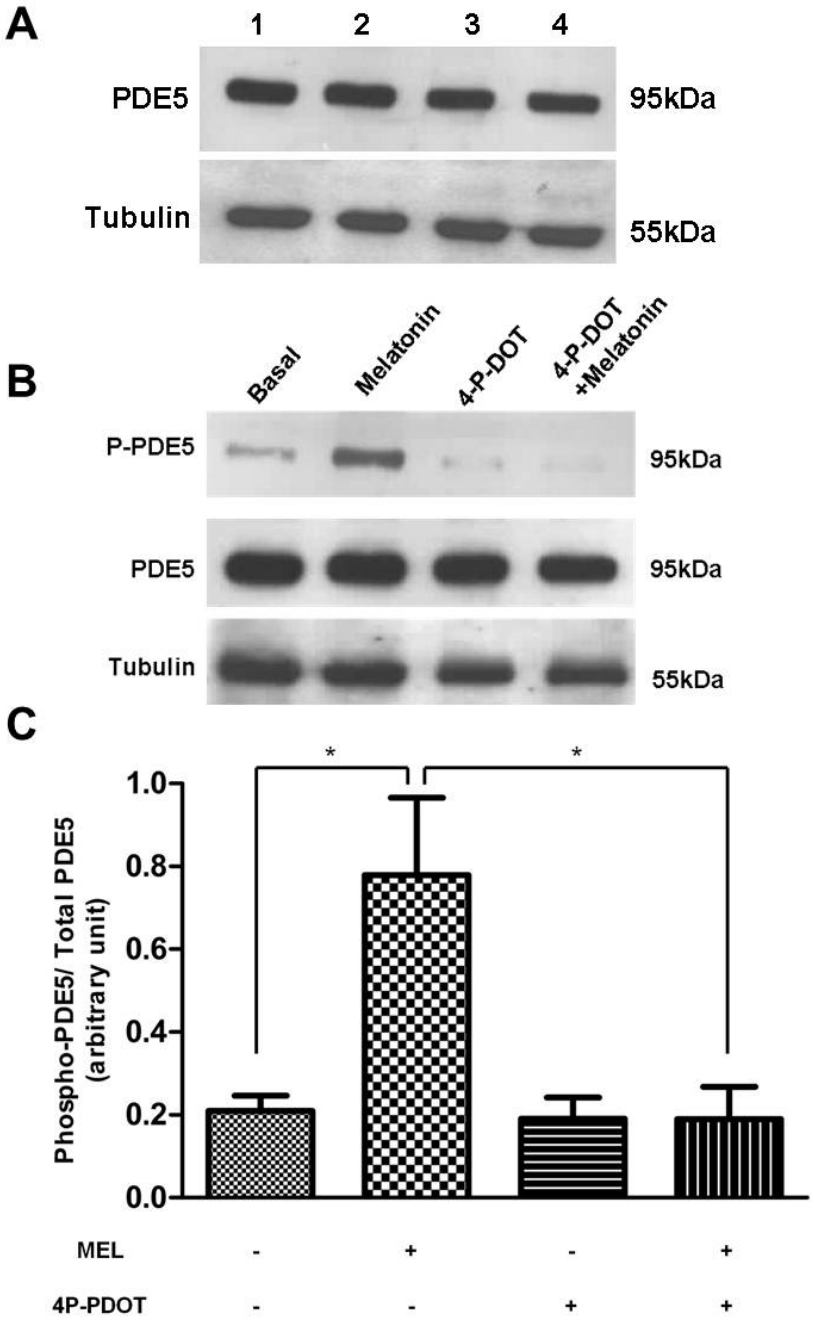


Figure 14. A: Phosphodiesterase-5 (PDE5) protein detected as an intense immunoreactive band at 95 kDa (Lanes 1-4) in porcine coronary artery homogenates from four different animals. B and C: Effect of melatonin on PDE5 phosphorylation in the presence and absence of the selective MT_2-receptor antagonist, 4P-PDOT (10^{-7} M). Panel C shows the immunodensity of Phospho-PDE5 (P-PDE5) normalized to the corresponding PDE5 protein band immunodensity * indicates a statistically significant difference between groups (P<0.05; ANOVA). (n = 6)

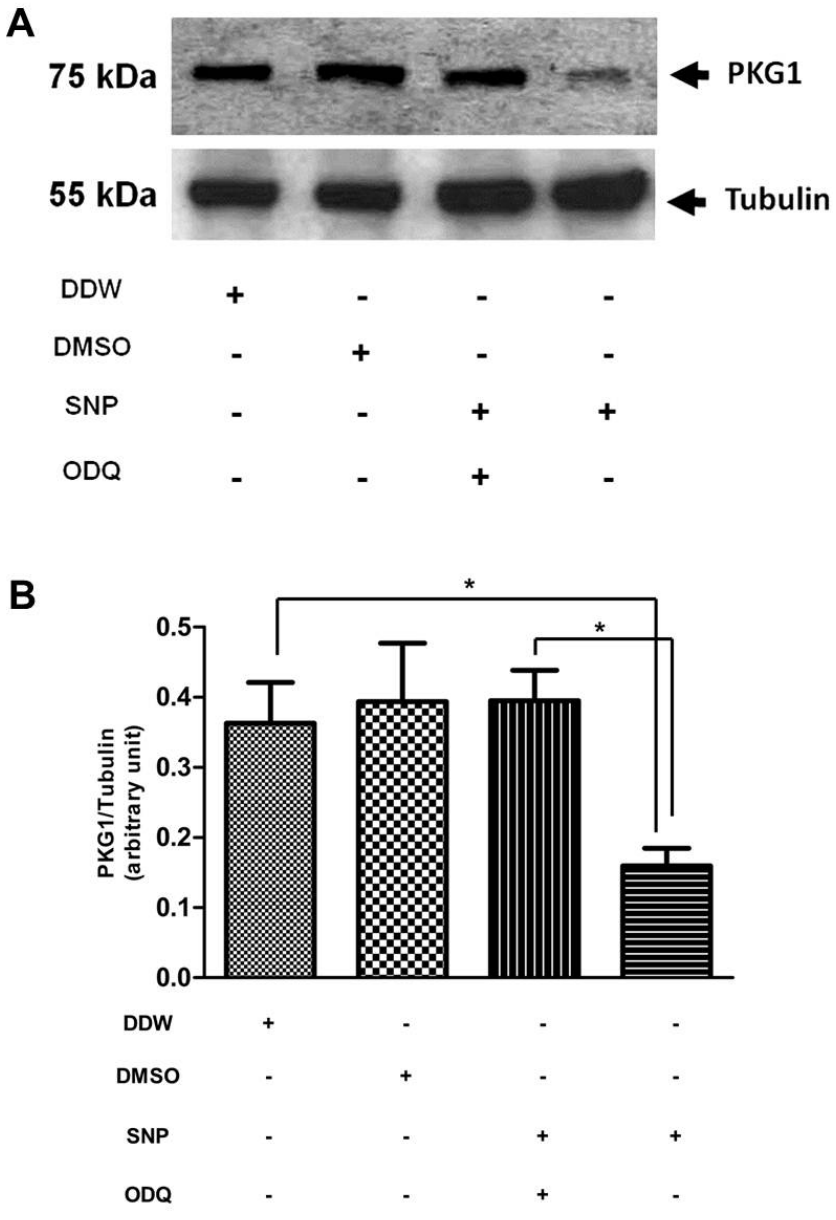


Figure 15. A and B: Effect of sodium nitroprusside or solvents (DDW or DMSO) on protein kinase-G1 (PKG1) protein expression in porcine coronary arteries, in the presence and absence of a soluble guanylyl cyclase inhibitor, ODQ (10^{-5} M). Panel B shows the immunodensity of PKG1 normalized to the corresponding β-tubulin protein band immunodensity. * indicates a statistically significant difference between groups (P<0.05; ANOVA). (n = 5)

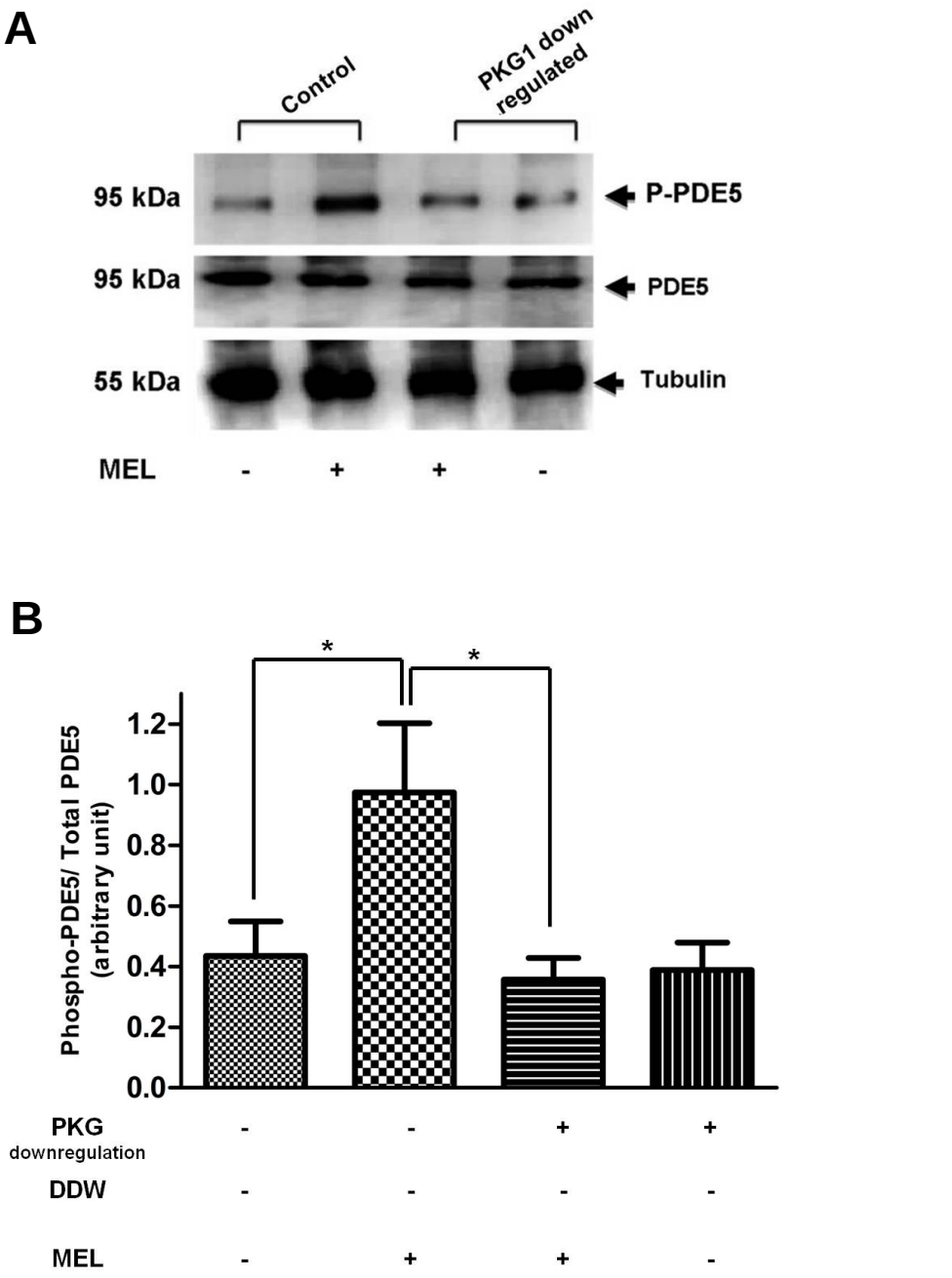


Figure 16. A and B: Melatonin-induced (10^{-7} M) PDE5 phosphorylation was significantly attenuated in porcine coronary arteries that were first treated with SNP (10^{-5} M; 24 hrs) to induce down regulation of PKG1. Panel B shows the immunodensity of phospho-PDE5 (P-PDE5) normalized to the corresponding PDE5 protein band immunodensity * indicates a statistically significant difference between groups (P<0.05; ANOVA). (n = 5).

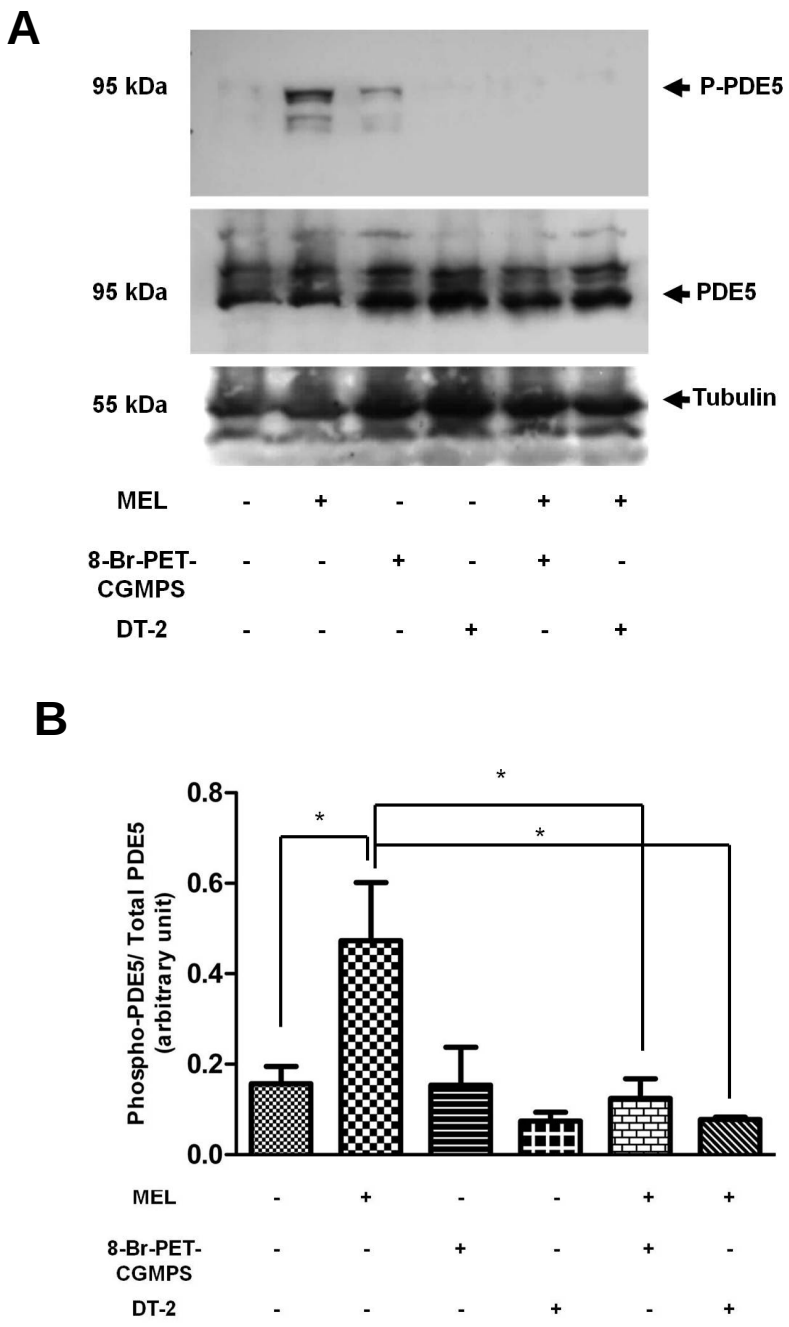


Figure 17. A and B: Melatonin-induced (10^{-7} M) PDE5 phosphorylation was significantly attenuated in porcine coronary arteries that were first treated with DT-2 (10^{-5} M; 30 minutes) or 8-Br-PET-CGMPS ($3x10^{-5}$ M; 30 minutes) to inhibit the PKG1 activity in the intact coronary artery rings. Panel B shows the immunodensity of phospho-PDE5 (P-PDE5) normalized to the corresponding PDE5 protein band immunodensity * indicates a statistically significant difference between groups (P<0.05; ANOVA). (n = 4)

The NO/cyclic GMP signaling pathway plays a central role in maintaining coronary vascular tone. In vascular smooth muscles cells, NO activates soluble guanylyl cyclase to stimulate the biosynthesis of cyclic GMP, which in turn activates cyclic GMP-dependent protein kinases (PKG) (Ignarro and Kadowitz, 1985; Ignarro, 1989). Activated PKG phosphorylates numerous ion channels and pumps, many of which promote a reduction in cytosolic calcium (Lincoln and Cornwell, 1991; Butt et al., 1993), membrane hyperpolarization and relaxation of arterial smooth muscle cells (Kannan and Johnson, 1995; Nelson and Quayle, 1995; Yamakage et al., 1996; Zhou et al., 1996). Impaired signaling via the NO/cyclic GMP pathway ultimately results in inhibition of NO-induced vascular smooth muscle relaxation and is associated with several cardiovascular disorders (e.g. hypertension, vasospasm, etc.) (Brunner et al., 2005; Vanhoutte, 2009).

In the present study, melatonin impaired porcine coronary arterial relaxation evoked by the NO donor, SNP (Figure 11A) but had no effect on the response to 8-Br-cGMP (Figure 11B), a stable, cell permeable analog of cyclic GMP that is resistant to PDE5-mediated hydrolysis (Corbin et al., 1986; Francis et al., 1988). The lack of effect of melatonin on 8-Br-cGMP-induced relaxations is consistent with the notion that melatonin increases degradation of intracellular cyclic GMP in response to NO, perhaps by increasing PDE5 catalytic activity. This hypothesis is further supported by the observation that the inhibitory effects of melatonin on both SNP-induced relaxation (Figure 12A) and increased intracellular cyclic GMP levels (Figure 13) were abolished in the presence of the selective PDE5 inhibitor, zaprinast, which strongly suggests a role for PDE5 in the inhibitory effect of melatonin.

In vascular smooth muscles cells, PDE5 activity is primarily regulated by its phosphorylation status (Surks et al., 1999; Sauzeau et al., 2000). Phosphorylation of PDE5 at serine-92 markedly enhances cyclic GMP binding affinity in the regulatory domain of PDE5 and increases the catalytic activity of the enzyme by several folds, leading to a decrease in intracellular cyclic GMP levels (Wyatt et al., 1998; Gopal et al., 2001; Murthy, 2001; Rybalkin et al., 2003). Since the inhibitory effects of melatonin on both SNP-induced relaxations (Figure 12A) and intracellular cyclic GMP accumulation (Figure 13) were abolished by the PDE5 inhibitor, zaprinast, I

hypothesized that an increase in PDE5 phosphorylation by melatonin may underlie the inhibitory effect on NO-induced responses. In support of this hypothesis, immunoblot analysis identified the expression of PDE5 protein in porcine coronary arteries (Figure 14A), and exposure to melatonin indeed caused a significant increase in PDE5 phosphorylation (Figure 14B-C). Moreover, PDE5 protein expression did not differ between the melatonin-treated and untreated tissues (Figure 14 A-C), indicating that the increase in PDE5 activity is the result of a posttranslational increase in PDE5 protein phosphorylation (Burns et al., 1992). That the stimulatory effect of melatonin on PDE5 phosphorylation was abolished in the presence of the MT_2-receptor antagonist, 4P-PDOT (Figure 14B-C), further supports the hypothesis that the impairment of NO-induced relaxation of porcine coronary arteries involves MT_2-receptor dependent stimulation of PDE5 phosphorylation and the ensuing decrease in intracellular cyclic GMP accumulation in response to NO. Although an effect of melatonin on soluble guanylyl cyclase, which together with PDE5 tightly regulates intracellular cyclic GMP levels in vascular smooth muscle (Wyatt et al., 1998), cannot be ruled out, the present findings clearly demonstrate a role for PDE5 in mediating the effects of melatonin on NO-induced responses in coronary arteries.

The exact signal transduction pathway by which MT_2-receptors are coupled to PDE5 phosphorylation in coronary artery smooth muscle cells remains to be elucidated. MT_2-receptors belong to the transducin family of G-protein coupled receptors; thus, one possibility is that the signal transduction pathway coupled to MT_2-receptors may be similar to that in the retinal rhodopsin-transducin ($G_{t\alpha\beta\gamma}$) system. Activation of the heterotrimeric G-protein, transducin ($G_{t\alpha\beta\gamma}$), leads to its dissociation into an active GTP bound alpha ($G_{t\alpha}GTP$) subunit, which in turn enhances PDE activity and results in a decrease in intracellular cyclic GMP levels (Artemyev, 1997; Natochin et al., 1997). Another possibility is that activation of MT_2-receptors by melatonin may result in activation of an intermediate signaling molecule (e.g. kinase) which then phosphorylates and activates PDE5. In vascular smooth muscles cells, PKG is the primary enzyme that phosphorylates PDE5 (Rybalkin et al., 2002). There are two families of PKG, i.e. PKG1 and PKG2, that are

derived from separate genes, *prkg1* and *prkg2* (Francis et al., 2010). In blood vessels PKG1 is the predominant form (Francis and Corbin, 1994; Pfeifer et al., 1998; Hofmann et al., 2000; Francis et al., 2010) and is more commonly involved in NO/cyclic GMP signaling (Francis et al., 2010). Hence, a pharmacological model of PKG1 downregulation was used to determine the role of PKG1 in the stimulatory effect of melatonin on PDE5 phosphorylation in porcine coronary arteries. Incubation of porcine coronary artery rings with SNP for 24 hrs caused a significant decrease in PKG1 protein expression, an effect that was abolished by the soluble guanylyl cyclase inhibitor, ODQ (Figure 15A-B), suggesting that elevated cyclic GMP levels present during continuous exposure to NO suppresses PKG1 expression. Notably, the melatonin-induced increase in PDE5 phosphorylation was nearly abolished in those arteries in which PKG1 expression was down-regulated (Figure16A-B). Similarly, pharmacologic blocked of PKG1 with two chemically unrelated selective PKG inhibitors i.e. DT2 (10^{-5} M; 30 min) (Dostmann et al., 2000; Dostmann et al., 2002; Taylor et al., 2004) or Rp-8-Br-PET-cGMPS ($3x10^{-5}$ M; 30 min) (Dou et al., 2008; Poppe et al., 2008; Zhang et al., 2012) significantly attenuated ($p<0.05$) the melatonin induced increase in PDE5 phosphorylation in porcine coronary arteries (Figure 17).Taken together, these results support the hypothesis that PKG1 activation mediates the stimulatory effect of melatonin on PDE5 phosphorylation in porcine coronary arteries.

3.5 Conclusion

In summary, the present study demonstrates that activation of MT_2-receptors increases PDE5 phosphorylation in coronary arteries, likely via activation of PKG1, which results in inhibition of NO-mediated increases in intracellular cyclic GMP and smooth muscle relaxation. Although our understanding of the role of specific melatonin receptor subtypes in cardiovascular homeostasis and disease is still evolving, the present findings shed new light on the interactions between melatonin and NO signaling in the vasculature.

CHAPTER 4. ROLE OF BK_{Ca} CHANNELS IN THE INHIBITORY EFFECT OF MELATONIN ON NO-INDUCED RELAXATIONS OF PORCINE CORONARY ARTERIES

4.1 Introduction

The large conductance, calcium-activated K channels (BK_{Ca}) are the predominant K channel expressed in coronary smooth muscle (Toro and Scornik, 1991). Owing to its large single channel conductance (approximately 150 pS under physiological conditions with respect to K^+ gradient) and high protein expression levels, BK_{Ca} plays a significant role in determining membrane potential of vascular smooth muscle cells including porcine coronary arteries (Trieschmann and Isenberg, 1989; Hill et al., 2010). BK_{Ca} is activated by both Ca^{2+} and voltage which favors its functioning as a dominant repolarizing current to act as a negative feedback control mechanism for contractile stimuli (Hill et al., 2010). Activation of BK_{Ca} occurs in response to focal Ca^{2+} release (Ca^{2+} spark) from the sarcoplasmic reticulum (Hill et al., 2010; Hill-Eubanks et al., 2011). These Ca^{2+} spark allow local Ca^{2+} concentrations below plasma membrane to reach levels approaching 10 μM for brief periods resulting in activation of a cluster of BK_{Ca} channels and K^+ efflux i.e. Spontaneous Transient Outward Currents (STOC) causing closure of voltage-dependent calcium channels and vasodilation (Benham and Bolton, 1986; Brayden and Nelson, 1992; Nelson et al., 1995; Hill et al., 2010; Hill-Eubanks et al., 2011).

NO-induced activation of BK_{Ca} channels leads to membrane hyperpolarization and an increased sensitivity of BK_{Ca} channels to Ca^{2+} (Sansom and Stockand, 1996). Membrane hyperpolarization closes voltage-dependent Ca^{2+} channels, reduces Ca^{2+} influx, and leads to a reduction in intracellular Ca^{2+} concentration and relaxation (Kannan and Johnson, 1995; Nelson and Quayle, 1995; Yamakage et al., 1996; Zhou et al., 1996). NO increases the formation of cGMP by stimulating sGC activity in vascular smooth muscle cells (Ignarro and Kadowitz, 1985; Ignarro, 1989). Cyclic GMP activates cGMP-dependent PKG, which phosphorylates several intracellular target proteins (Lincoln and Cornwell, 1991; Butt et al., 1993). PKG-induced phosphorylation of

BK_{Ca} channels stimulates channel activity and increases K^+ efflux (Alioua et al., 1998; Fukao et al., 1999). In addition to this indirect cGMP-PKG dependent activation of BK_{Ca} channels (Robertson et al., 1993; Zhou et al., 1996; Sansom et al., 1997), the direct activation of BK_{Ca} channels (Bolotina et al., 1994) or by a combination of both direct and indirect mechanisms (Peng et al., 1996) by NO, has also been proposed. The observation that the selective inhibitor of sGC, ODQ (10^{-5} M) completely abolished NO-induced relaxation (Figure 18A) and increase in intracellular cGMP (Figure 18B) strongly suggests that cGMP-dependent (or indirect) activation of BK_{Ca} channels is involved in NO-induced relaxation of porcine coronary arteries. This argument is further supported by the studies demonstrating that the NO-cGMP-PKG pathway induces smooth muscle relaxation by a BK_{Ca} channel–dependent mechanism in porcine coronary arteries (White et al., 1995).

In organ chamber studies, blockade of BK_{Ca} channels with iberiotoxin (10^{-7}M; IbTx), a potent and selective BK_{Ca} channel blocker (Wallner et al., 1995) caused a significant rightward shift in the concentration-response curve for SNP (10^{-9}-10^{-5}M) (Figure 19) (pD_2=7.73 ± 0.1 for SNP vs pD_2=6.89 ± 0.1 in the presence of IbTx, $P < 0.05$) supporting a crucial role for BK_{Ca} channels in NO-induced relaxation of porcine coronary arteries. In functional vascular reactivity studies, at concentrations similar to that used in this study, melatonin causes melatonin-receptor dependent inhibition of BK_{Ca} channel activity in small and large diameter arteries (Geary et al., 1997; Geary et al., 1998). Together with the observation that melatonin (10^{-7} M) inhibited the NO-induced increase in intracellular cGMP levels, these data led me to hypothesize that the impairment of NO-induced relaxation of porcine coronary arteries by melatonin involves MT_2 receptor-dependent inhibition of NO-activated BK_{Ca} channel activity in porcine coronary smooth muscle cells. Therefore, the objectives of the present study were (1) to determine if melatonin inhibits the whole-cell BK_{Ca} currents activated by NO in freshly dissociated porcine coronary smooth muscle cells and (2) to elucidate the role of MT_2-receptor activation in the inhibitory effect of melatonin on NO-activated BK_{Ca} currents.

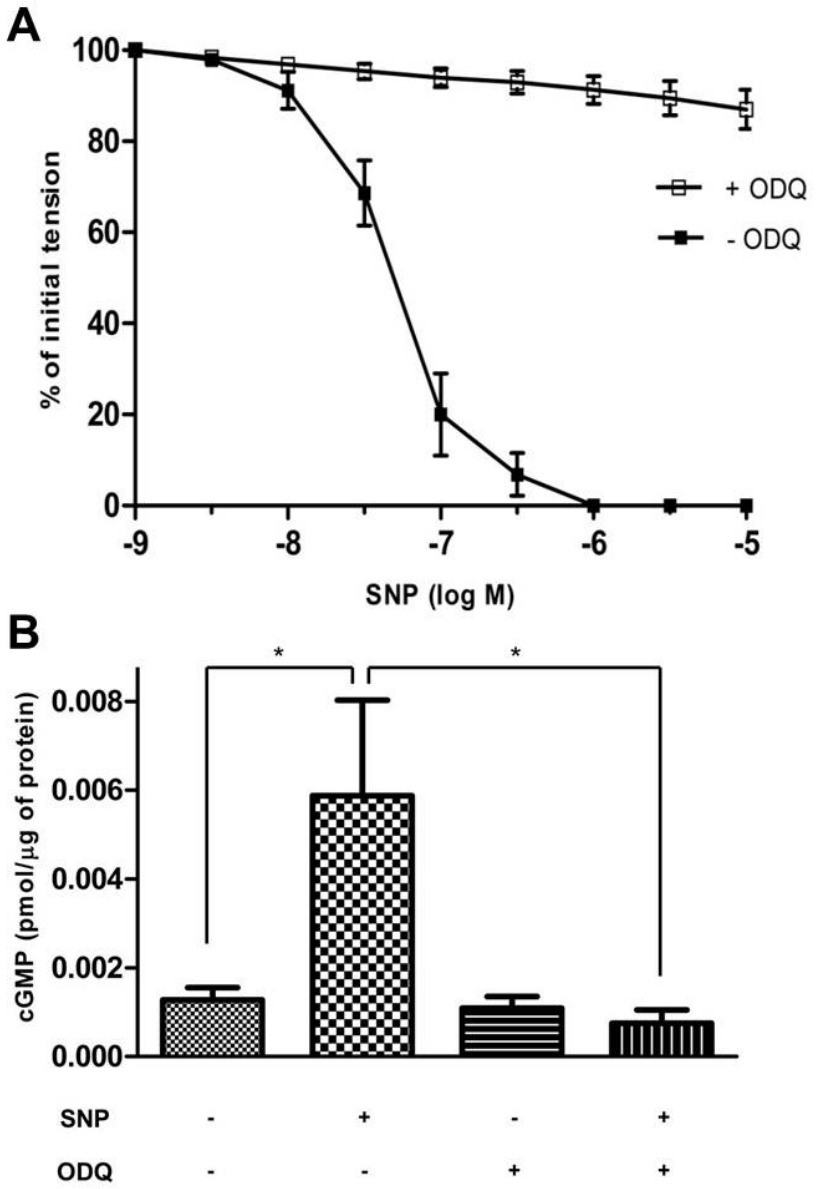


Figure 18. Effect of ODQ (10^{-5} M), a soluble guanylyl cyclase inhibitor, on NO-induced relaxations of isolated porcine coronary arteries (A) and increases in intracellular cGMP levels in isolated porcine coronary arteries (B) (without endothelium). A: Data are expressed as a percentage of the initial increase in tension induced by U46619 ($1–3 \times 10^{-9}$ M), which did not differ significantly between control rings and control rings incubated with ODQ. B: Data are expressed as pico mole of cGMP per microgram of the total protein present in the supernatant of the tissue homogenates. * indicates a statistically significant difference in the presence of SNP ($P<0.05$; ANOVA). Each point represents the mean ± S.E.M. (n = 4-5).

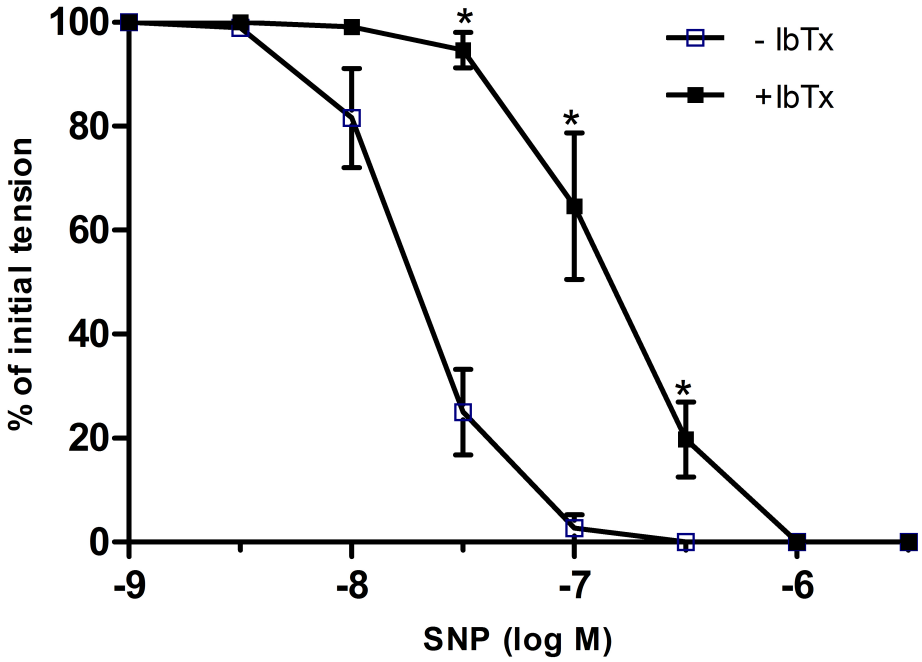


Figure 19. Effect of iberiotoxin (IbTx, 10^{-7} M) on sodium nitroprusside (SNP)-induced relaxations of isolated porcine coronary arteries (without endothelium). Data are expressed as a percentage of the initial increase in tension induced by U46619 (1–3 × 10^{-9} M), which did not differ significantly between control rings and control rings incubated with melatonin. * indicates a statistically significant difference between treatment groups (P <0.05; Student t-test). Each point represents the mean ± S.E.M. (n = 6-7).

4.2 Materials and methods

4.2.1 Materials

The following drugs were used in the study: 4-aminopyridine, DL-Dithiothreitol, NS1619, melatonin, Sodium nitroprusside, (Sigma, St. Louis, MO), iberiotoxin, 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), 4P-PDOT (4-Phenyl-2-propionamidotetralin), 8-Br-cGMP, zaprinast (Tocris, Ellisville, MO). Drug solutions were prepared daily, kept on ice, and protected from light until used. NS1619 and zaprinast were prepared as 10-mM stock solutions with DMSO, and serial dilutions were prepared in bath solution. 4P-PDOT and melatonin were prepared as 10 mM stock solution with ethanol and serial dilutions were prepared in bath solution. Iberiotoxin, Sodium nitroprusside, 4-aminopyridine and 8-Br-cGMP stock solution was prepared with distilled water.

The composition of the physiological salt solution was as follows (in mM): 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, and 10 glucose (pH=7.4).

4.2.2 Methods

4.2.2.1 Pharmacologic studies

Coronary artery relaxation was studied in endothelium-denuded rings contracted with U46619 (1-3 x 10^{-9} M) as described in chapter 2, section 2.2.2.1 and 4. After the U46619-induced contraction reached a stable plateau, concentrations-response curve for sodium nitroprusside (SNP; 10^{-9} to 10^{-5} M) were obtained in the absence and presence of iberiotoxin (10^{-7} M) or ODQ (10^{-5} M), which was added to the bath solution 20-30 minutes prior to contraction with U46619 and remained in contact with the tissues throughout the remainder of the experiment. Four to eight coronary arterial rings were prepared from each heart. In some rings, the endothelium was removed by gently rubbing the intimal surface with a fine forceps.

4.2.2.2 Cyclic GMP measurements

Coronary artery rings were pretreated with ODQ (20 min, 10^{-5} M) before exposing to SNP (5 min, 10^{-5} M). After drug treatments, rings were frozen in liquid nitrogen and cGMP content was determined as described in the chapter 3, section 3.2.2.3 and expressed as pmol/µg of protein.

4.2.2.3 Immunoblot detection of BK_{Ca} channel subunits α and β

Coronary arteries were immediately frozen in liquid nitrogen after preparation. For detection of BK_{Ca} channel subunits (i.e. α and β), coronary artery tissues were homogenized with an IKA® Ultra, Turrax-T8 homogenizer (IKA works. Inc., Wilmington, NC, USA) at 4˚C in lysis buffer, supplemented with a protease and phosphatase inhibitor cocktail (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The tissue homogenates were kept on ice for 10 min and afterwards centrifuged for 10 min at 10,000g. Supernatants were collected for protein determination and Western blotting. Protein concentration was assayed using a Bio-Rad D_c Protein assay kit (Biorad Laboratories, Hercules, CA, USA). Aliquots of supernatants containing equal amounts of protein (20-100 µg) were separated on either 7.5% (α-subunit) or 12% (β-subunit)

polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins were electrophoretically transferred onto a PVDF membrane (Biorad Laboratories, Hercules, CA, USA). Blots were blocked with 5% nonfat dry milk in phosphate buffered saline (PBS, pH 7.4) and then incubated overnight at 4˚C with a primary antibody specific for BK_{Ca} α-subunit (Alomone Labs, Ltd., Israel) or β-subunit (Santa Cruz Biotechnology) using a dilution of 1:5000. The membranes were then probed with a horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology). To ensure equal loading, the blots were analyzed for either β-tubulin or actin expression. Immunodetection was performed using an enhanced chemiluminescence light detecting kit (Thermo Scientific, Rockford, IL, USA). The images are representative of 4 different samples. I observed a very strong signal for the BK_{Ca} β-subunits in porcine coronary artery homogenates. Therefore to optimize the signal and minimize the signal to background ratio, I loaded relatively small amount of protein (20 µg) for detection of β-subunits. The BK_{Ca} α-subunits signals were optimum at 100 µg protein loading.

4.2.2.4 Vascular smooth muscle cell (SMC) isolation

Porcine hearts were obtained from a local slaughterhouse. The left circumflex and left anterior descending coronary arteries were isolated and excess fat and connective tissues were removed. Coronary arteries were cut into small pieces in low calcium Tyrodes's solution of the following composition (in mM): 145 NaCl, 4 KCl, 0.05 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (pH=7.4 adjusted with NaOH) at 4 ºC. Subsequently the tissue pieces were incubated for 15 minutes with gently shaking at 37 ºC in 1 ml of the low calcium Tyrodes's solution containing 1.5 mg/ml papain (14 U/mg) and 1 mg/ml DL-Dithiothreitol (DDT), followed by incubation for 15 minutes at 37 ºC in 2 ml of the low calcium Tyrodes's solution containing 2 mg/ml collagenase (196 U/ml), 0.5 mg/ml elastase (90 U/ml), and 1 mg/ml soybean trypsin inhibitor (10,000 U/ml). The enzyme solutions were removed either by centrifugation at 500g for 1-2 minutes or by removing the supernatant with a pipette. Then 5 ml of fresh low calcium Tyrodes's solution was added to tissue pieces. The single vascular smooth muscle cells were released by gently triturating with a fire polished pasetur pipette or 5 ml glass pipette and collected in supernatant. The cells in supernatant

were spun down at 500g for 5 min, resuspended in fresh low calcium Tyrodes's solution, and stored at 4 ºC. Cells were used within 5-8 hrs of isolation.

4.2.2.5 Immunofluorescence staining of porcine coronary SMCs

Single drops of the freshly isolated porcine coronary vascular smooth muscle cell suspensions were placed on glass slides. The cells were allowed to attach to the glass surface at room temperature for 20-30 minutes followed by washing (3 X 5 min) with PBS (pH 7.4) and fixing with ice cold methanol (-20 ˚C) for 10 min. The fixed cells were air dried, washed with wash solution (PBS, pH 7.4 containing 0.1% of Triton X-100; 3 X 5 min) and non--specific antibody binding was blocked with a blocking solution containing 5% nonfat dry milk and 3% Bovine serum albumin (BSA) in wash solution. Subsequently cells were incubated overnight at 4℃ in blocking solution containing primary antibody specific for either smooth muscle cell actin (SMCA) (1:250; Santa Cruz Biotechnology Inc., USA) or BK_{Ca} α- (1:150; Alomone Labs, Ltd., Israel) or β-subunit (1:250; Santa Cruz Biotechnology). For co-localization of the BK_{Ca} α- or β-subunit with SMCA, double immunofluorescent staining was performed by incubating the cells with more than one primary antibody at the same time. Detection of the primary antibodies against the BK_{Ca} α- and β-subunit or SMCA was accomplished using goat antirabbit Alexa Fluor 488-conjugated and goat antimouse Alexa Fluor 568-conjugated (Life technologies, Grand Island, NY, USA) secondary antibody, respectively. Cells incubated without primary antibody, with rabbit or mouse serum or rabbit or mouse IgG were included as negative controls. Slides were drained by blotting with filter paper and a drop of mounting medium containing DAPI, a nuclear stain and an anti-fade reagent (Vector Laboratories, Inc., Burlingame, CA, USA) was added to the slides. The images were obtained using an Olympus confocal laser-scanning microscope. The images were generated using FV300 (v. 4.3) Confocal Software and Adobe Photoshop 5.5.

4.2.2.6 Electrophysiology/patch clamping studies

The freshly isolated vascular smooth muscle cells were placed in a small Nunclon™ plastic Petri dish constantly perfused with the extracellular solution (in M) 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, and 10 glucose (pH=7.4 adjusted with NaOH). The whole-cell voltage clamp

technique was used for recording BK_{Ca} current. Micropipettes (2-5 MΩ resistance) were made from capillary tubing (WPI, Sarasota, FL) using a programmable puller (P-80/PC; Sutter Instruments, Novato, CA). Series resistance and capacitance compensation were adjusted maximally using a voltage clamp amplifier (Axopatch 200A, Axon Instrument, Union City, CA). Data were digitized at 2 kHz, filtered at 1 kHz. The pipette solution was composed of (in M) 145 potassium aspartate, 5 NaCl, 1 $CaCl_2$, 2.2 EGTA, 10 HEPES, and 7.5 glucose (pH=7.4 adjusted with KOH). Drugs were dissolved in extracellular solution as 10 mM stocks and diluted to desired concentrations in extracellular solution. Cells were held at -60 mV and BK_{Ca} currents were recorded during 200 ms depolarizing voltage steps between -70 to +60 mV in 10 mV increments. Interpulse duration was 3 s. Current amplitude was measured as the mean current during the last 100 ms of voltage steps and plotted against the membrane voltage.

To identify BK_{Ca} currents, whole cell K currents were measured in the absence or presence of IbTx (10^{-7} M) and/or 4-AP (10^{-3} M). To determine the effects of melatonin on voltage or NO-activated BK_{Ca} currents a range of concentrations of melatonin (10^{-9}-10^{-7} M) were applied either consecutively or separately, in the presence or absence of SNP (10^{-5} M). To determine the role of MT_2-receptors, and PDE5 in the inhibitory effect of melatonin on NO-activated BK_{Ca} currents, cells were pretreated for 5-10 minutes, either with the MT_2-receptor antagonist, 4P-PDOT (10^{-7} M) or PDE inhibitor, zaprinast (10^{-5} M) before exposing cells to melatonin. In a separate series of experiments the effect of melatonin (10^{-7} M) were recorded on the BK_{Ca} currents elicited by 8-Br-cGMP (10^{-3} M), a stable, cell permeable analog of cyclic GMP. In addition, BK_{Ca} currents activated by SNP were recorded in the absence and presence of ODQ (10^{-5} M). Current-voltage (I /V) plots were determined for each drug tested.

4.2.2.7 Data analysis

Membrane capacitance was calculated by integrating capacitive currents generated by 10 mV hyperpolarizing pulse after electronic cancellation of the pipette-patch capacitance, and peak K^+ current amplitudes were expressed in picoamperes per picofarad (pA/pF) to normalize for differences in cell membrane area between isolated vascular smooth muscle cells. Data were

analyzed by using pCLAMP software (Axon Instruments). In western blotting, immuno-fluorescence and relaxations studies, *n* refers to the number of animals from which blood vessels were taken. In patch clamp studies *n* refers to the number of freshly dissociated vascular smooth muscle cells which were isolated from at least 3-4 different animals. Values were compared by Student's *t*-test for paired or unpaired observations, or by analysis of variance to determine significance between groups, as appropriate. Results are expressed as mean ± S.E. and values were considered to be significantly different when $P < 0.05$.

4.3 Results

4.3.1 Expression of BK_{Ca} channel subunits in porcine coronary arteries

Western immunoblot analysis revealed the presence of BK_{Ca} α- and β-subunits in porcine coronary arteries. A strong immunoreactive band at 125 kDa or 30 kDa corresponding to BK_{Ca} α-(Figure 20A, lanes 1-2) or β-(Figure 1B, lanes 1-2) subunits was detected in immunoblots of porcine coronary artery homogenates. The BK_{Ca} α- and β-subunits protein immunoreactivity was abolished when the primary antibody was replaced with non-immune IgG followed by incubation with the secondary antibody (data not shown).

4.3.2 Immunofluorescence staining of SMCs

The immunofluorescent staining of freshly isolated porcine coronary vascular smooth muscle cells (SMCs) showed the expression of BK_{Ca} β- and α- subunits (Figure 21C and 22C). The SMCs were identified by staining cells for actin (red fluorescence, Figure 21B and 22B), a specific marker of vascular smooth muscle cells. The BK_{Ca} β- and α-subunits were co-localized with the actin (yellow fluorescence, Figure 21D and 22D). Incubation of SMCs with non-immune IgG followed by the secondary antibody resulted in a complete lack of staining (Figure 22-23, B and C, inset).

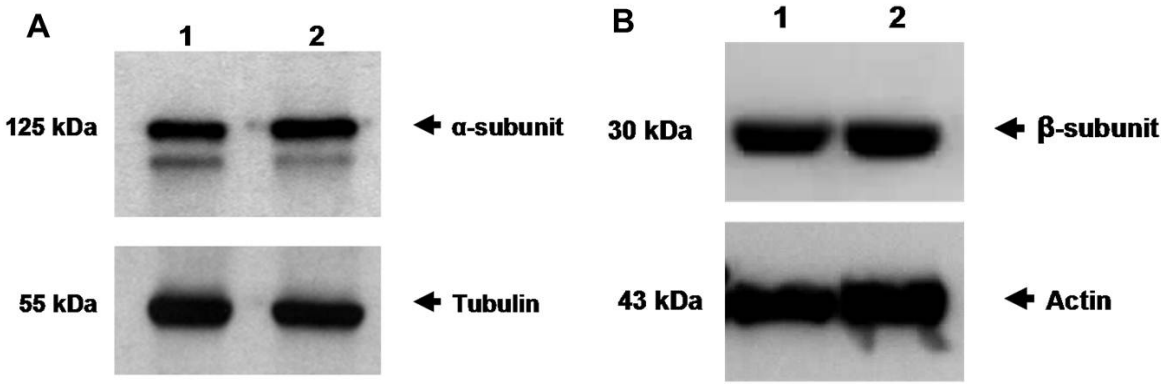


Figure 20. Representative western blot images of the BK_{Ca} α- (panel A) and β (panel B)-subunits protein in porcine coronary artery homogenates from 2 animals (lanes 1-2). Immunoreactive bands at 125 kDa and 30 kDa corresponds to BK_{Ca} α- and β-subunits protein. The figure is representative of images taken from arteries of four different animals (n = 4).

4.3.3 Electrophysiology/patch clamping studies

4.3.3.1 Identification of BK_{Ca} currents in coronary SMCs

The whole-cell patch configuration with 200-ms voltage steps from a holding potential (V_h) of −60 mV to test potentials in the range of−70 to +60 mV in 10 mV increments were used to activate whole-cell K currents in freshly isolated porcine coronary SMCs. Iberiotoxin (10^{-7} M), a potent and selective BK_{Ca} channel blocker (Wallner et al., 1995) inhibited (60 ± 7% at +30 mV; n=6, $P<0.05$) a large component of the voltage activated whole-cell macroscopic K currents compared to control (Figure 23A-B). The co-application of 4-aminopyridine, a non-selective voltage-dependent K-channel blocker (Mathie et al., 1998) almost completely inhibited (95 ± 03% at +30 mV compare to control; n=5, $P<0.05$) the whole-cell K currents (Figure 23A-B). These data suggests that the BK_{Ca} current is the predominant K current species in porcine coronary SMCs.

4.3.3.2 Role of BK_{Ca} in NO-induced relaxations of coronary arteries

In organ chamber studies, blockade of BK_{Ca} channels with iberiotoxin (10^{-7}M) caused a significant rightward shift (Figure 19) (pD_2=7.73 ± 0.1 for SNP vs pD_2=6.89 ± 0.1 in the presence of IbTx, $P < 0.05$) in the concentration-response curve for SNP (10^{-9}-10^{-5}M) supporting a crucial role for BK_{Ca} channels in NO-induced relaxation of porcine coronary artery.

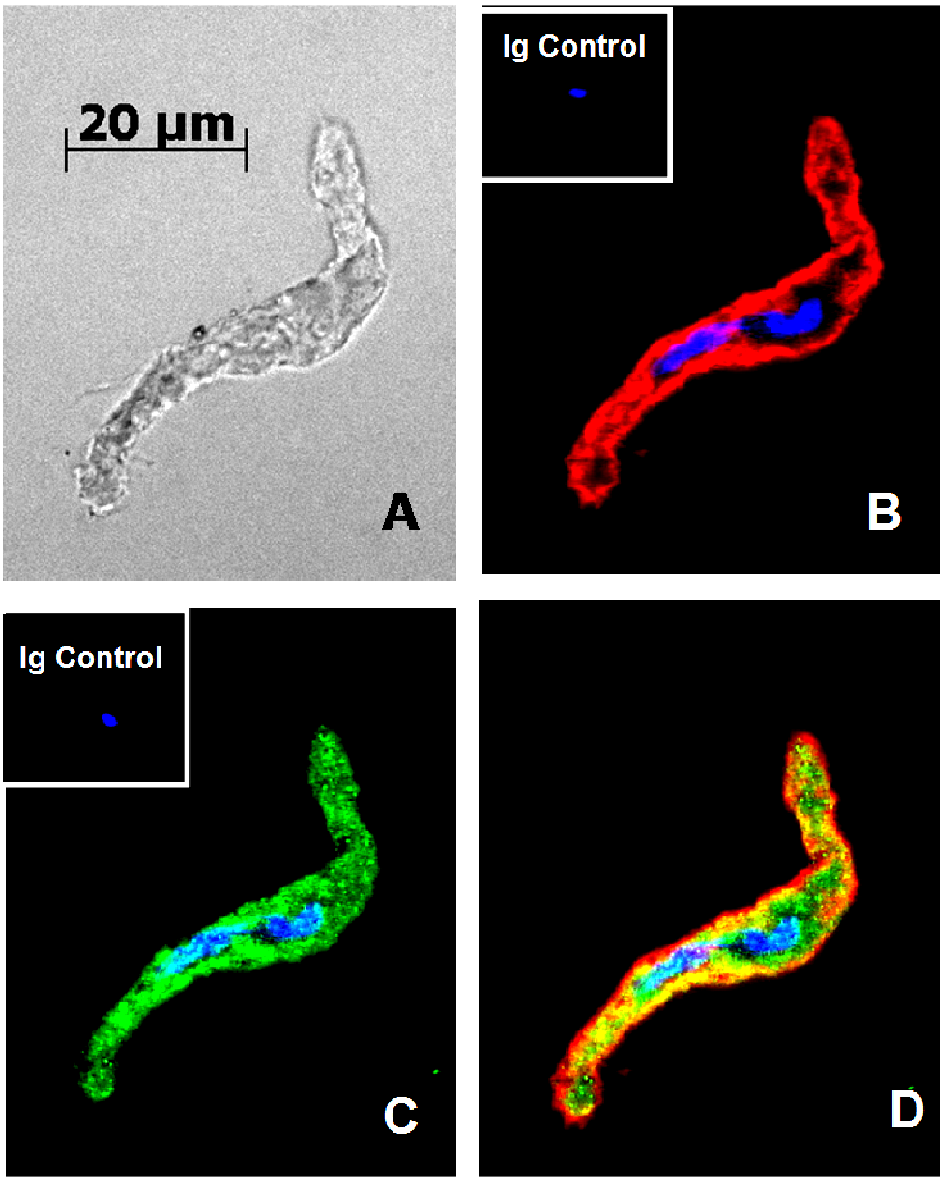


Figure 21. Representative images of immunofluorescent co-localization of smooth muscle cell actin (SMCA) and BK_{Ca} β-subunit in freshly isolated porcine coronary vascular smooth muscle cells. (A) image taken using DIC microscopy, (B) SMCA and Cell Nucleus – red and blue fluorescence, respectively, (C) BK_{Ca} β-subunit – green fluorescence, , (D) co-localized areas showing yellow fluorescence; insert in (B) and (C) demonstrates lack of staining after incubation of SMCs with non-immune IgG followed by the secondary antibody. Bar = 20 µM. *n* refers to the number of animals from which SMCs were isolated for the immunostaining. (n = 4).

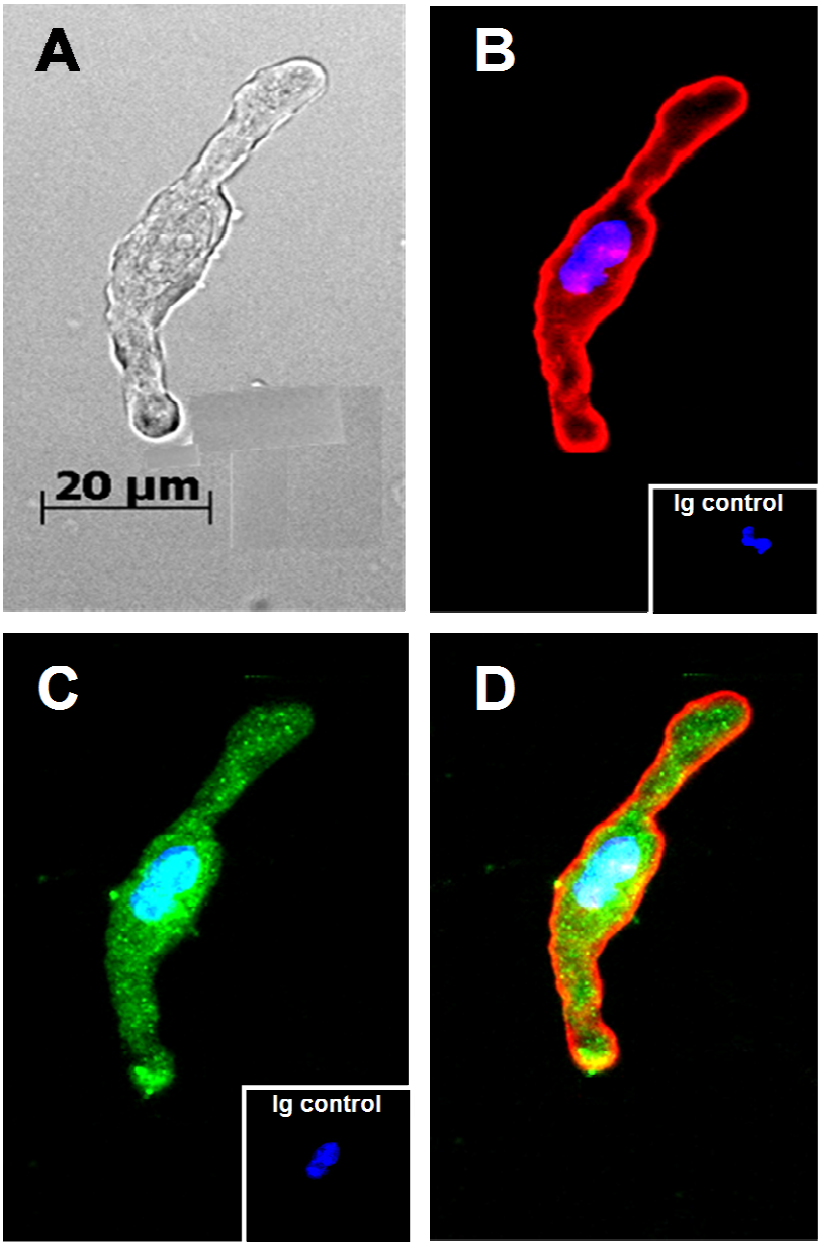


Figure 22. Representative images of immunofluorescent co-localization of smooth muscle cell actin (SMCA) and BK_{Ca} α-subunit in freshly isolated porcine coronary vascular smooth muscle cells. (A) image taken using DIC microscopy, (B) SMCA and Cell Nucleus – red and blue fluorescence, respectively, (C) BK_{Ca} α-subunit – green fluorescence, (D) co-localized areas showing yellow fluorescence; insert in (B) and (C) demonstrates lack of staining after incubation of SMCs with non-immune IgG followed by the secondary antibody. Bar = 20 µM. *n* refers to the number of animals from which SMCs were isolated for the immunostaining. (n = 4).

Similar results were obtained in patch clamp studies. Addition of SNP (10^{-5} M), caused a robust increase (*P<0*.05) in outward K currents in porcine coronary SMCs compared to control (Figure 24). Blockade of the BK_{Ca} channels with IbTx (10^{-7} M) significantly inhibited (64 ± 12% at +30 mV compare to SNP; n=6, *P<0*.05) the NO-induced increase in whole-cell K currents (Figure 24) thus supporting an important role of BK_{Ca} channel function in NO-induced relaxation of porcine coronary arteries.

4.3.3.3 NO-induced activation of BK_{Ca} channels is cGMP-dependent

External application of SNP (10^{-5} M), an exogenous NO-donor (Kowaluk et al., 1992), caused a robust increase in whole-cell BK_{Ca} currents in porcine coronary SMCs (Figure 24 and 25A). The blocked of sGC with ODQ (10^{-5} M) (Garthwaite et al., 1995), completely abolished the SNP (10^{-5} M)-induced increase in BK_{Ca} currents (Figure 25B) suggesting that NO-induced activation of BK_{Ca} currents is cGMP-dependent. Moreover, pretreatment of coronary arterial rings with ODQ (10^{-5} M), abolished both the NO-induced relaxation (Figure 18A) and increase in intracellular cGMP levels (Figure 18B). Taken together, these results strongly support the view that the cGMP-dependent activation of BK_{Ca} channels mediates the NO-induced relaxation of porcine coronary arteries.

4.3.3.4 Melatonin inhibits NO-activated BK_{Ca} currents via MT_2-receptors

SNP (10^{-5} M) an NO-donor (Kowaluk et al., 1992), (Figure 24 and 25A) and 8-Br-cGMP (10^{-3} M), (Figure 27) a stable, cell permeable analog of cyclic GMP (Corbin et al., 1986; Francis et al., 1988)**,** each caused a significant increase in BK_{Ca} currents in coronary SMCs (*P* < 0.05). Co-application of melatonin (10^{-7} M) significantly and reversibly reduced, the BK_{Ca} currents activated by SNP (Figure 26A-B) but not by 8-Br-cGMP (Figure 27). At +30 mV test potential, melatonin reduced the NO-activated BK_{Ca} current amplitude to 48.86 ± 5% of SNP control (n=7, *P* < 0.05), and after 5 min washout the currents recovered to 71.84 ± 5% of control (n=12) (Figure 26A-B). Melatonin-induced inhibition of NO-activated BK_{Ca} currents was blocked by pretreatment (and co-application) of SMCs with 4-P-PDOT (10^{-7} M), a specific MT_2 receptor antagonist (Dubocovich, 1988; Zlotos, 2005). Figure 28A and B shows that no suppression (*P* > 0.05) of the BK_{Ca} currents

was observed at all test potentials, when 4-P-PDOT (10^{-7} M) was co-applied with melatonin (10^{-7} M) and SNP (10^{-5} M). The average current obtained at +30 mV test potential after 5 min perfusion of melatonin and SNP along with 4-P-PDOT was 94.41 ± 14% of SNP (n=6, $P < 0.05$) (Figure 28A and B), suggesting that the inhibition of BK_{Ca} currents by melatonin was mediated by MT_2 receptors. The BK_{Ca} currents activated by voltage steps or SNP were unaffected by the presence of antagonist alone.

4.3.3.5 Role of PDE5 in the inhibitory effect of melatonin on BK_{Ca}

Pretreatment of coronary SMCs with zaprinast (10^{-5} M), a cyclic GMP specific PDE5 inhibitor (Mullershausen et al., 2001; Bender and Beavo, 2006), abolished the inhibitory effect of melatonin on NO-activated BK_{Ca} currents (Figure 29). The average current obtained at +30 mV test potential after 5 min perfusion of melatonin along with SNP and zaprinast was 97.38 ± 12.5% of SNP (n=6, $P < 0.05$) (Figure 29), suggesting that the inhibition of BK_{Ca} currents by melatonin involves PDE5 activation. These results are consistent with the observation that blocked of the cyclic GMP specific PDE with zaprinast (10^{-5} M) (Mullershausen et al., 2001; Bender and Beavo, 2006), abolishes the inhibitory effects of melatonin on both the NO-induced relaxation of coronary arteries (Figure 12A) and increase in intracellular cGMP levels (Figure 13).

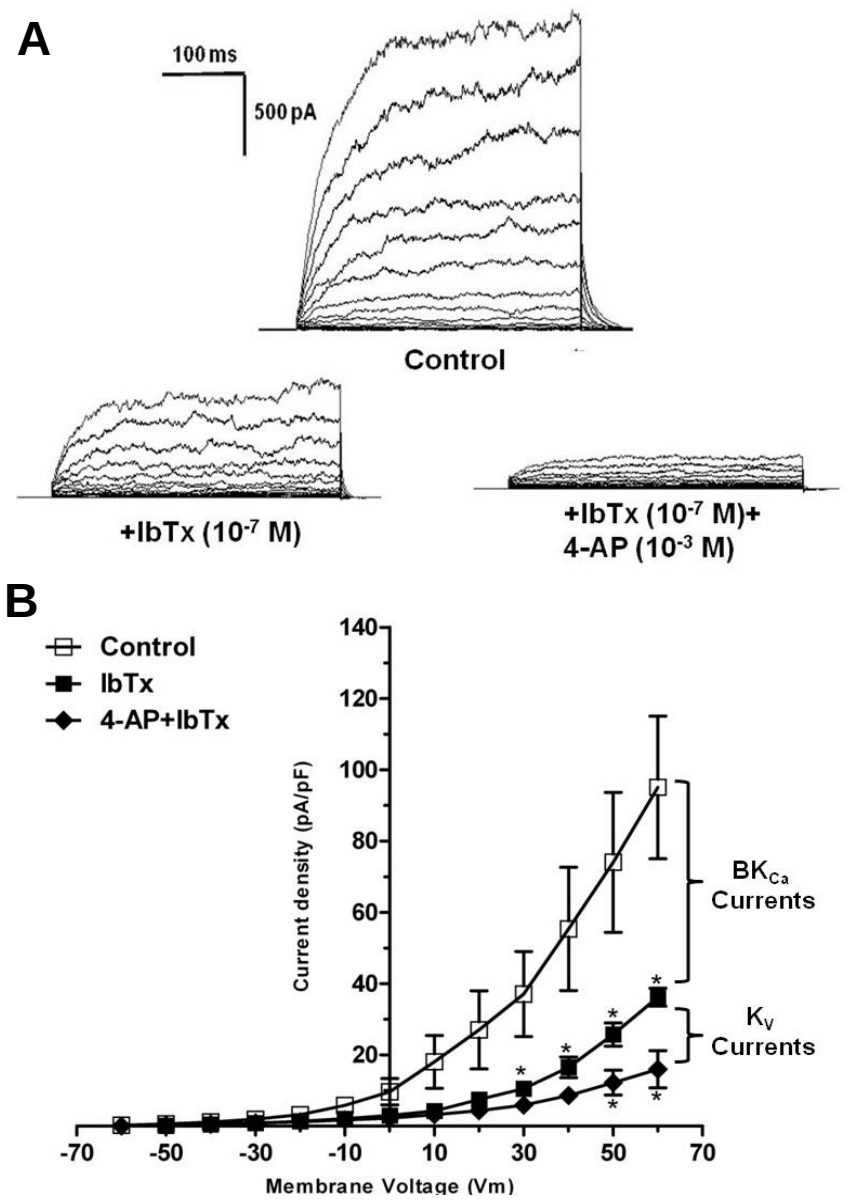


Figure 23. The predominant currents recorded in freshly isolated SMCs from porcine coronary arteries show the characteristics of BK_{Ca} channel. (A) Representative current traces displaying blockade by Iberiotoxin (IbTx; 10^{-7} M) and/or 4-Aminopyridine (4-AP, 10^{-3} M) of a large component of the outward current compared with control (B) I–V relationships of membrane currents in SMCs revealing the inhibitory effect of IbTx (10^{-7} M) and/or 4-AP (10^{-3} M) on whole cell macroscopic K^+ currents. * indicates a statistically significant difference between control and IbTx group (*P* <0.05; ANOVA). Each point represents the mean ± S.E.M. (n = 6).

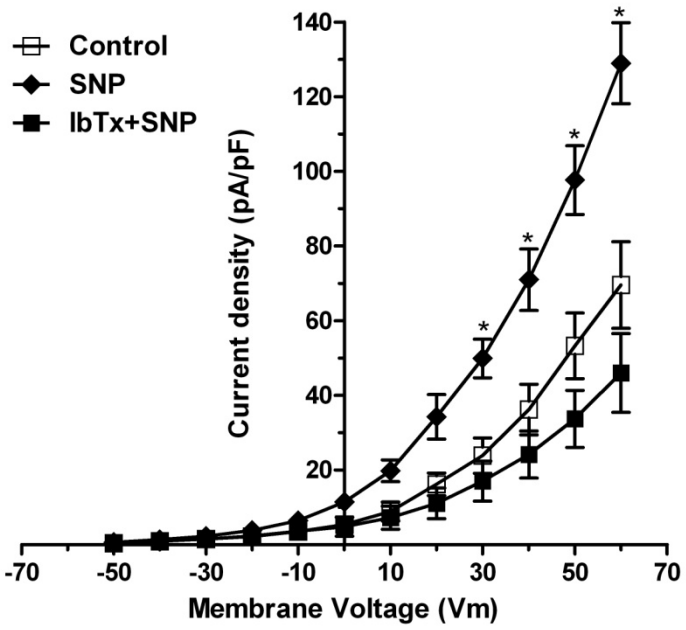


Figure 24. The I–V relationships of membrane currents in freshly isolated SMCs from porcine coronary arteries revealing the inhibitory effect of IbTx (10^{-7} M) on whole cell macroscopic K^+ currents activated by sodium nitroprusside (SNP, 10^{-5} M). * indicates a statistically significant difference between control and SNP group (*P* <0.05; ANOVA). Each point represents the mean ± S.E.M. (n = 6).

4.4 Discussion

The results of this study indicate that melatonin acting via MT_2-receptors increases the PDE5 activity, resulting in inhibition of NO/cGMP-dependent activation of BK_{Ca} channels, and impaired NO-induced vasorelaxation. These conclusions are supported by the findings that: 1) BK_{Ca} α- and β-channel subunits are expressed in porcine coronary arteries and in freshly dissociated coronary SMCs; 2) iberiotoxin significantly inhibited the NO-induced relaxation of coronary arteries and increase in whole cell BK_{Ca} currents in coronary SMCs thus supporting an important functional role of BK_{Ca} channel in coronary artery relaxation in response to NO; 3) melatonin inhibited BK_{Ca} currents evoked by NO, but not by 8-Br-cGMP in coronary smooth muscle cells; 4) blockade of MT_2 receptors abolished the inhibitory effect of melatonin on the NO-induced increase in BK_{Ca} current, thus supporting a role for MT_2-receptors; and 5) inhibition of PDE5 with

zaprinast, abolished the inhibitory effect of melatonin on the NO-induced increase in BK_{Ca} current, suggesting a mechanistic coupling between MT_2-receptor activation and PDE5 activity.

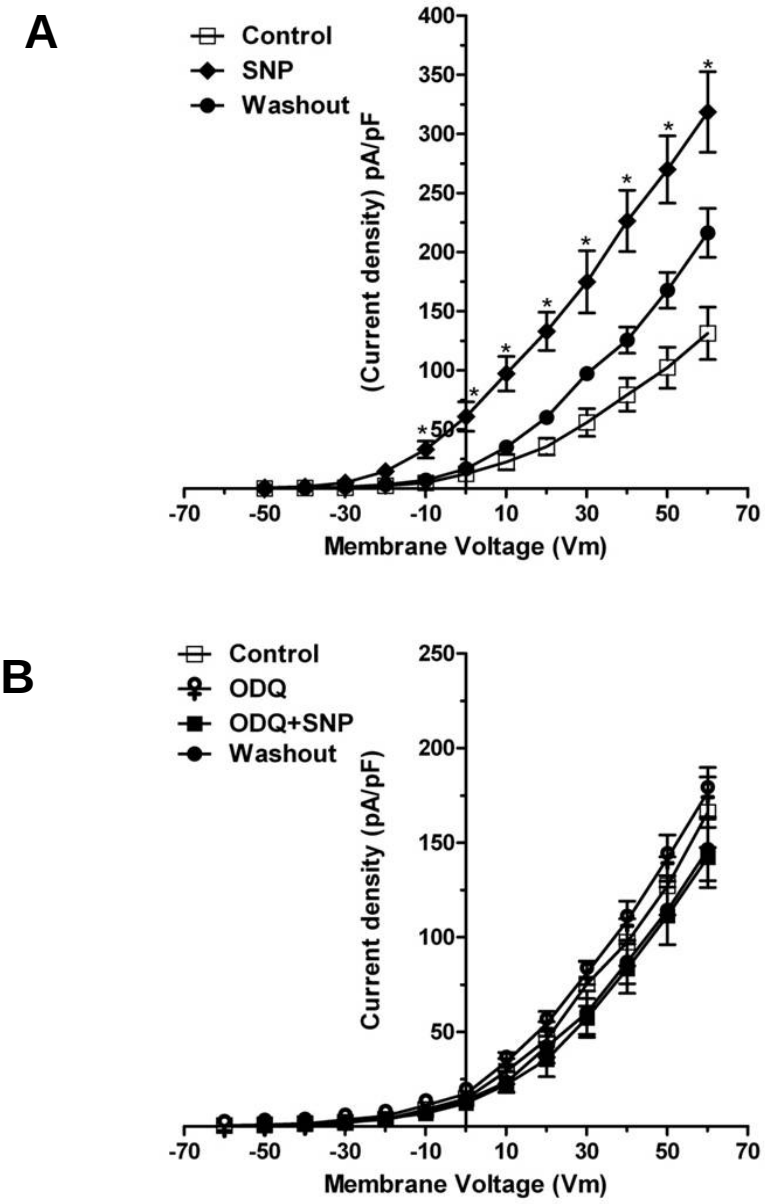


Figure 25. SNP (10^{-5}M) an exogenous NO-donor causes significant increase in whole-cell BK_{Ca} currents (A) in freshly isolated porcine coronary SMCs which were abolished in the presence of ODQ (10^{-5}M) a soluble guanylyl cyclase inhibitor (B). * indicates a statistically significant difference between control and SNP group (P <0.05; ANOVA). Each point represents the mean ± S.E.M. (n=5).

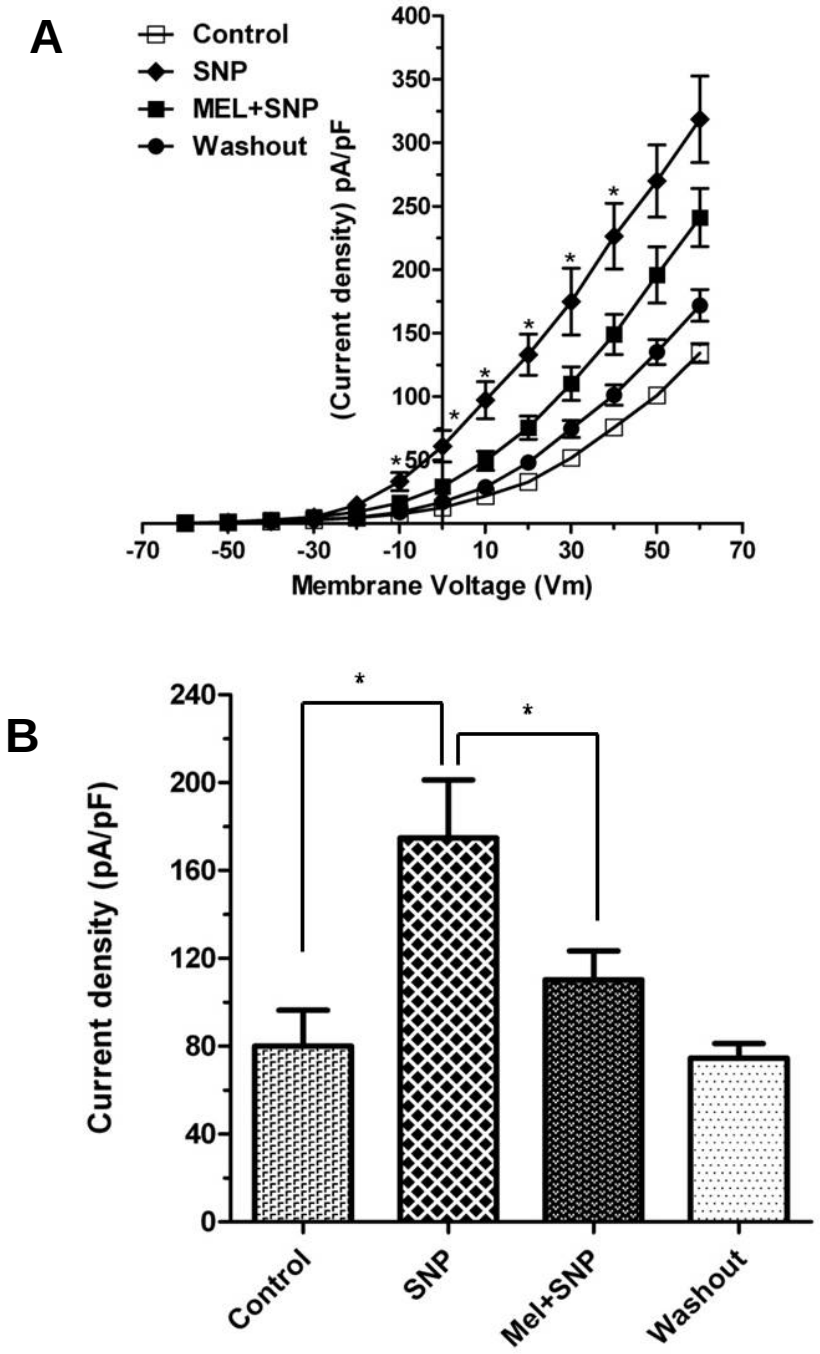


Figure 26. Melatonin (MEL; 10^{-7} M) inhibits whole cell macroscopic K^+ currents activated by NO (A & B) in freshly isolated SMCs from porcine coronary arteries. A**:** I/V relationship; B: normalized currents elicited at +30 mV step. * indicates a statistically significant difference between SNP and MEL+SNP group groups ($P < 0.05$; ANOVA). Each point represents the mean ± S.E.M. (n=7).

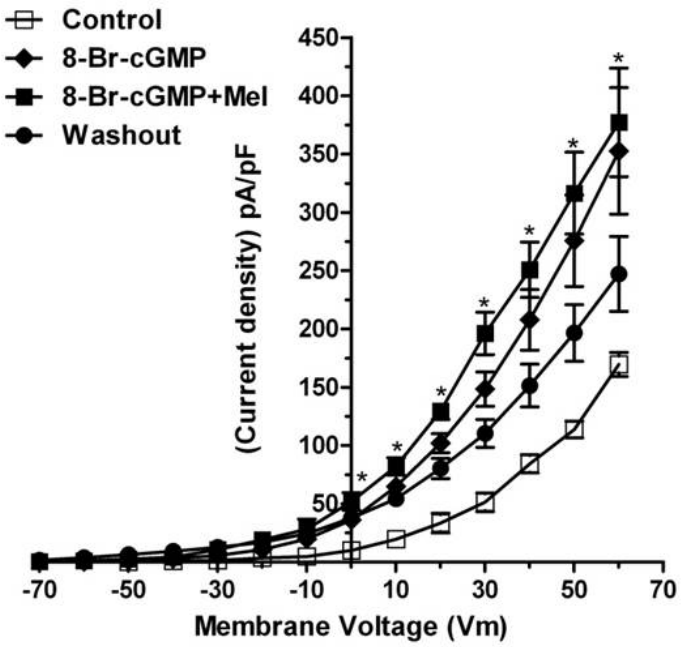


Figure 27. Melatonin (MEL; 10^{-7} M) has no effect on whole cell macroscopic K^+ currents activated by 8-Bromo-cGMP, a stable cell permeable analogue of cGMP. * indicates a statistically significant difference between control and 8-Br-cGMP group ($P < 0.05$; ANOVA). Each point represents the mean ± S.E.M. (n = 5-6).

The results further indicate that the cGMP-dependent activation of BK_{Ca} channels mediates NO-induced relaxation of coronary arteries, since the NO-induced relaxation of coronary arteries, increase in intracellular cyclic GMP accumulation, and whole cell BK_{Ca} currents in coronary SMCs are all blocked by ODQ, a soluble guanylyl cyclase inhibitor (Garthwaite et al., 1995). These findings for the first time provide a direct evidence for the role of the BK_{Ca} channels in the inhibitory action of melatonin on NO-induced vasorelaxation and provide new insight into potential mechanisms by which melatonin may regulate blood vessel diameter.

Owing to the high density expression and large conductance (Toro and Scornik, 1991), BK_{Ca} channels plays an important role in setting and maintaining the membrane potential of coronary arteries (Trieschmann and Isenberg, 1989; Hill et al., 2010). NO activates soluble guanylyl cyclase to stimulate the biosynthesis of cyclic GMP, which in turn activates cyclic GMP-dependent protein kinases (PKG) (Ignarro and Kadowitz, 1985; Ignarro, 1989).

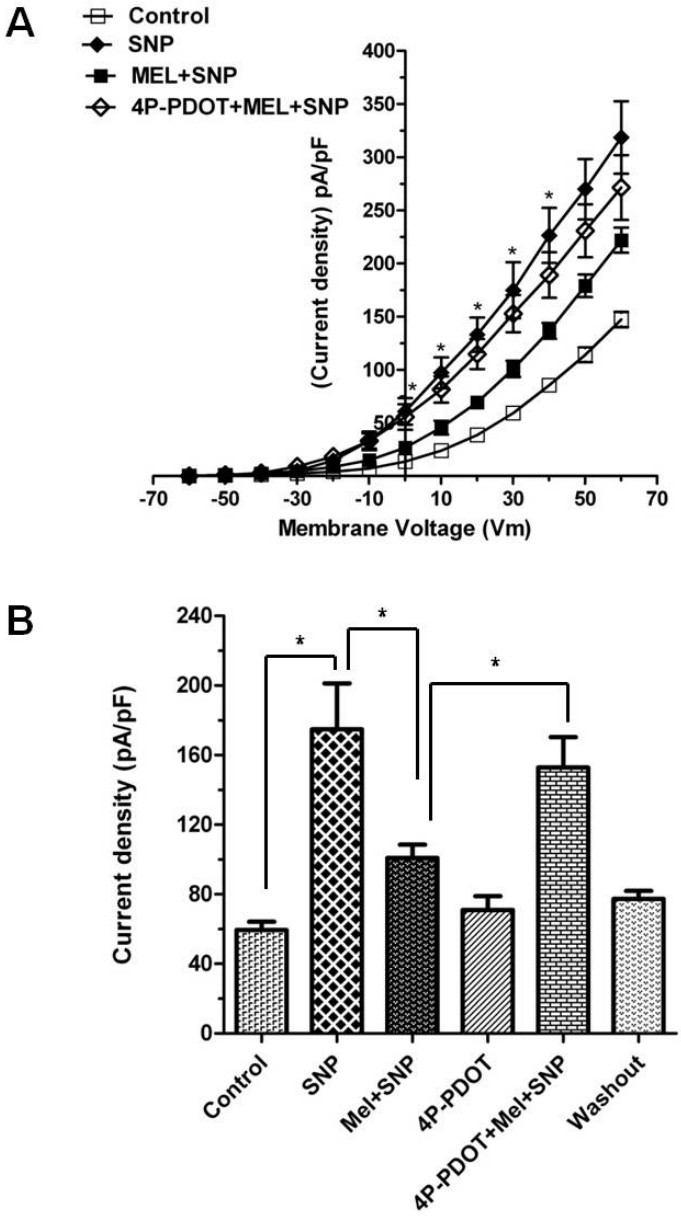


Figure 28. Pretreatment of the freshly isolated SMCs from porcine coronary arteries with the MT_2-receptor antagonist, 4-PPDOT (10^{-7} M) abolishes the inhibitory action of melatonin on whole cell macroscopic K^+ currents activated by NO. A: I/V relationship; B: normalized currents elicited at +30 mV step. * indicates a statistically significant difference between control and SNP, SNP and MEL+SNP, MEL+SNP and 4P-PDOT+MEL+SNP groups (*P* <0.05; ANOVA). Each point represents the mean ± S.E.M. (n=6-7).

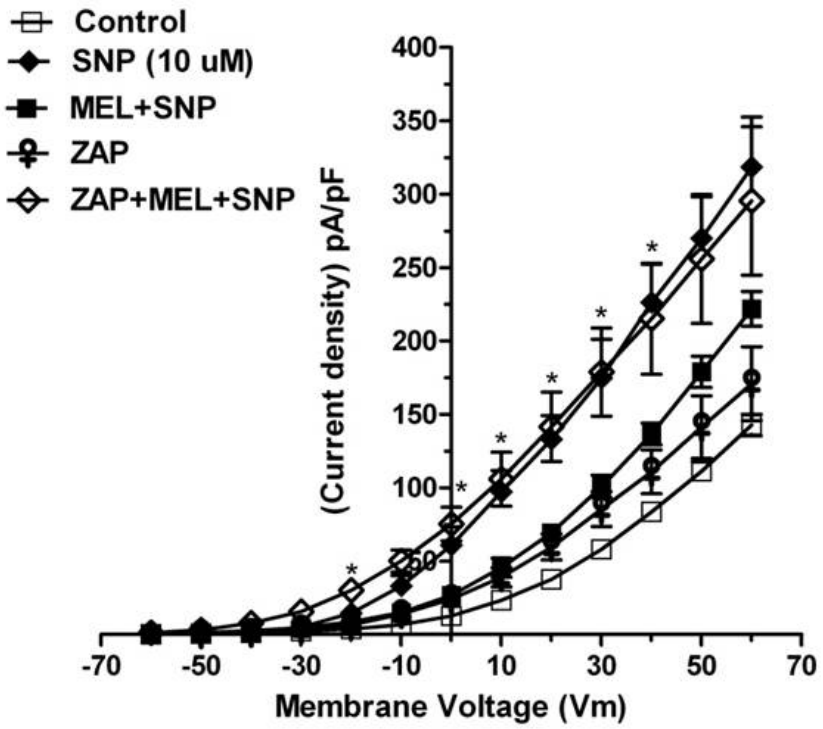


Figure 29. Pretreatment of the freshly isolated SMCs from porcine coronary arteries with the cGMP-specific PDE5 inhibitor, zaprinast (ZAP) (10^{-5} M), abolishes the inhibitory action of melatonin on whole cell macroscopic K^+ currents activated by NO. * indicates a statistically significant difference between Control and SNP, MEL+SNP and ZAP+MEL+SNP groups (*P* <0.05; ANOVA). Each point represents the mean ± S.E.M. (n = 6-7).

Activated PKG phosphorylates numerous ion channels and pumps, many of which promote a reduction in cytosolic calcium (Lincoln and Cornwell, 1991; Butt et al., 1993). In particular, PKG activates BK_{Ca} channels (Alioua et al., 1998; Fukao et al., 1999), which hyperpolarizes the arterial smooth muscle cell closing the voltage-dependent Ca^{2+} channels, and consequential reduction in intracellular Ca^{2+} concentration and relaxation (Kannan and Johnson, 1995; Nelson and Quayle, 1995; Yamakage et al., 1996; Zhou et al., 1996). Therefore, an alteration in BK_{Ca} channel function may impair the vascular smooth muscle relaxation in response to NO and has been implicated in pathogenesis of cardiovascular disorders (Rusch, 2009).

In the present study, immunoblotting and immunohistochemical studies identified a robust expression of BK_{Ca} α- and β-subunits in both intact coronary arteries (Figure 20A,B) and freshly

dissociated coronary SMCs (Figure 21-22), respectively. In the functional organ chamber studies, blockade of BK_{Ca} channels with iberiotoxin causes a significant rightward shift in the concentration-response curve (CRC) for the SNP, an exogenous NO-donor (Figure 19) (Kowaluk et al., 1992), suggesting a crucial role of BK_{Ca} channel function in NO-induced relaxation of coronary arteries. Multiple mechanisms by which NO activates the BK_{Ca} channel, has been proposed including cGMP-dependent or indirect activation (Robertson et al., 1993; Zhou et al., 1996; Sansom et al., 1997), direct activation (Bolotina et al., 1994) or a combination of both direct and indirect mechanisms (Peng et al., 1996). The observation that the selective inhibitor of sGC, ODQ (Garthwaite et al., 1995) completely abolished the NO-induced relaxation of coronary arteries (Figure 18A); increase in intracellular cGMP accumulation (Figure 18B) and BK_{Ca} currents in coronary SMCs (Figure 25B) strongly suggests that cGMP-dependent activation of BK_{Ca} channels mediates relaxation of porcine coronary arteries.

Using functional vascular reactivity studies Geary provided indirect evidence that melatonin at concentrations similar to that used in this study, causes melatonin-receptor dependent inhibition of BK_{Ca} channel activity in small and large diameter arteries from the rats (Geary et al., 1997; Geary et al., 1998). However, the study did not provide any direct electrophysiological evidence. Moreover, a recent study reported that melatonin inhibits the TEA-sensitive K^+ current via activation of MT_2 receptors expressed on rat rod-dominant ON type bipolar cells (Rod-ON-BCs) in rat retinal slices (Yang et al., 2011). Up to now, there is no direct electrophysiological evidence to support the involvement of BK_{Ca} channels in the inhibitory effect of melatonin on vascular tone. Together with the observation that melatonin inhibited the NO-induced increase in intracellular cGMP levels (Tunstall et al., 2011), I hypothesize that the melatonin impairs the NO-induced relaxation of coronary arteries by inhibiting the NO-dependent activation of BK_{Ca} channels in coronary SMCs.

In patch clamp studies, melatonin inhibited BK_{Ca} currents evoked by NO (Figure 26A-B), but not by 8-Br-cGMP (Figure 27) in coronary smooth muscle cells. The blockade of MT_2-receptors with 4P-PDOT (10^{-7} M), a selective MT_2-receptor antagonists, (Dubocovich, 1988; Zlotos, 2005), abolished the inhibitory effect of melatonin on BK_{Ca} activation induced by SNP (Figure 28A-B), thus

supporting a role for MT_2-receptors. The lack of the effect of melatonin (10^{-7} M) on the BK_{Ca} currents induced by 8-Br-cGMP, a stable, cell permeable analog of cyclic GMP that is resistant to PDE5-mediated hydrolysis (Corbin et al., 1986; Francis et al., 1988) is consistent with the notion that melatonin increases degradation of intracellular cyclic GMP in response to NO, perhaps by increasing PDE5 catalytic activity. This hypothesis is further supported by the observation that the inhibitory effects of melatonin on BK_{Ca} activation were abolished in the presence of the selective PDE5 inhibitor, zaprinast (Figure 29), which strongly suggests a role for PDE5 in the inhibitory effect of melatonin. Taken together, these results suggest a mechanistic coupling between MT_2-receptor activation and increase in PDE5 activity with ensuing decrease in intracellular cyclic GMP accumulation and NO/cyclic GMP-dependent BK_{Ca} activation.

The exact signal transduction pathway by which MT_2-receptors are coupled to the inhibition of BK_{Ca} channel in coronary artery smooth muscle cells remains to be elucidated. However, the present study clearly demonstrates that melatonin inhibits the NO/cyclic GMP-dependent activation of BK_{Ca} channels which is dependent on MT_2-receptors coupled increase in PDE5 catalytic activity in coronary SMCs. One plausible mechanism is that activation of MT_2-receptors by melatonin may result in activation of an intermediate signaling molecule (e.g. kinase) which then phosphorylates and activates PDE5 resulting in increase cGMP degradation and inhibition of BK_{Ca} channel activation. In support of this hypothesis, I have demonstrated that melatonin stimulates the PDE5 phosphorylation (Figure 14) and that either downregulation (Figure 15) or the blocked of PKG1 (Figure 17) significantly attenuates the melatonin-induced increase in PDE5 phosphorylation in intact coronary arteries (Figure 16-17). Moreover, several studies (Benitez-King et al., 2001; Soto-Vega et al., 2004) demonstrated that melatonin indeed stimulate the protein kinase C (PKC) activity in various cell types and that the activation of PKC results in increased PKG activity both *in vitro* and *in vivo* (Hou et al., 2003). Thus, activation of MT_2-receptors by melatonin may result in activation of PKC which in turn stimulates the PKG1 activity resulting in the increase PDE5 phosphorylation and activity and inhibition of the NO/cyclic GMP-dependent activation of BK_{Ca} channels. Interestingly, the PKC-dependent inhibition of protein phosphatase 1 is known to

stimulate PDE5 phosphorylation in gastrointestinal smooth muscles, along with a corresponding decrease in intracellular cGMP accumulation in response to NO (Murthy, 2008), suggesting yet another potential pathway by which MT_2-receptor activation may be coupled to inhibition of the BK_{Ca} channel activity.

4.5 Conclusion

In summary, the results of the present study demonstrates that melatonin increases PDE5-mediated cGMP degradation by activating MT_2-receptors, resulting in inhibition of BK_{Ca} channel activation in response to NO and impaired NO-induced vasorelaxation. Since BK_{Ca} channels function as a dominant repolarizing current in vascular smooth muscle cells, melatonin may potentially increase the vascular tone by causing membrane depolarization-induced influx of calcium secondary to inhibition of BK_{Ca} channels.

CHAPTER 5. SUMMARY

Several studies have demonstrated the expression of specific melatonin receptors in different vascular beds, however very few studies have explained the physiological function of vascular melatonin receptors. Understanding the function of vascular melatonin receptors is both physiologically and clinically significant as MT_1 and MT_2-receptors are expressed in human coronary arteries and their expression is altered in cardiovascular diseases. Moreover, the widespread and unregulated use of melatonin as a dietary supplement to treat sleep disorders further warrants a thorough examination of the potential effects of melatonin on the coronary circulation. To this end, I have focused on an important research question of identifying the specific melatonin receptor subtype and elucidating the cellular and molecular mechanism mediating the inhibitory effect of melatonin on NO-cGMP signaling pathway in coronary artery. The results suggests that the MT_2-receptors coupled stimulation of PDE5 catalytic activity with resultant decrease in intracellular cGMP levels and inhibition of BK_{Ca} channels function in coronary smooth muscle cells underlies the inhibitory effect of melatonin on NO-induced relaxations of coronary arteries.

It is now recognized that specific receptors for melatonin are present in the arteries of many species, including humans. Three distinct melatonin receptor subtypes mediate the physiological effects of melatonin: MT_1- and MT_2-receptors, which are members of the transmembrane G-protein-coupled-receptor family, and MT_3-receptors, which have been identified as the enzyme quinone reductase 2. Previous studies from our laboratory reported that melatonin impairs NO-induced relaxations of isolated coronary arteries by activity specific melatonin receptors. However, the pharmacological identity of the specific melatonin receptor mediating the inhibitory effect of melatonin on coronary vascular tones in not known.

The results of the present study demonstrate the presence of functional MT_2-receptors on porcine coronary arterial smooth muscle cells, and that activation of these receptors by melatonin inhibits the ability of the smooth muscle to relax in response to NO. In contrast to melatonin, 5-MCA-NAT, a selective *MT_3*-receptor agonist had no effect on NO-induced relaxation of isolated

coronary arteries, suggesting that the inhibitory effect of melatonin does not involve activation of *MT_3*-receptors. However, pretreatment of coronary arterial rings with the selective MT_2-receptor antagonist, 4P-PDOT or luzindole abolished the inhibitory effect of melatonin on NO-induced relaxation of coronary arteries, thus supporting a role of MT_2-receptors in the inhibitory effect of melatonin on NO-induced relaxation of coronary arteries.

Since the major mechanism by which NO relaxes smooth muscle is by increasing intracellular cyclic GMP levels, I explored the possibility that the activation of MT_2-receptors by melatonin attenuates the NO-induced increase in cyclic GMP levels in coronary smooth muscle. Indeed, pretreatment of coronary arterial rings with melatonin inhibited the SNP-induced increase in cyclic GMP. Moreover, the inhibitory effect of melatonin on cyclic GMP accumulation was abolished by incubation of the tissues with the MT_2-receptor antagonist, 4P-PDOT supporting the view that MT_2-receptors activation mediates the inhibitory effect of melatonin on cyclic GMP accumulation in response to NO.

The cGMP-specific phosphodiesterase type-5 (PDE5) degrades the cyclic GMP and acts as major negative regulator of NO/cyclic GMP signaling. In vascular smooth muscles cells, PDE5 activity is primarily regulated by its phosphorylation status, whereby phosphorylation of PDE5 at serine-92 markedly enhances the catalytic activity of the enzyme. At present, the role of PDE5, if any, in the modulation of cyclic GMP levels by melatonin in vascular smooth muscle is not known. Therefore, I tested the hypothesis that melatonin activates PDE5 in coronary arteries, thereby decreasing intracellular cyclic GMP accumulation in response to NO and inhibiting NO-induced relaxation.

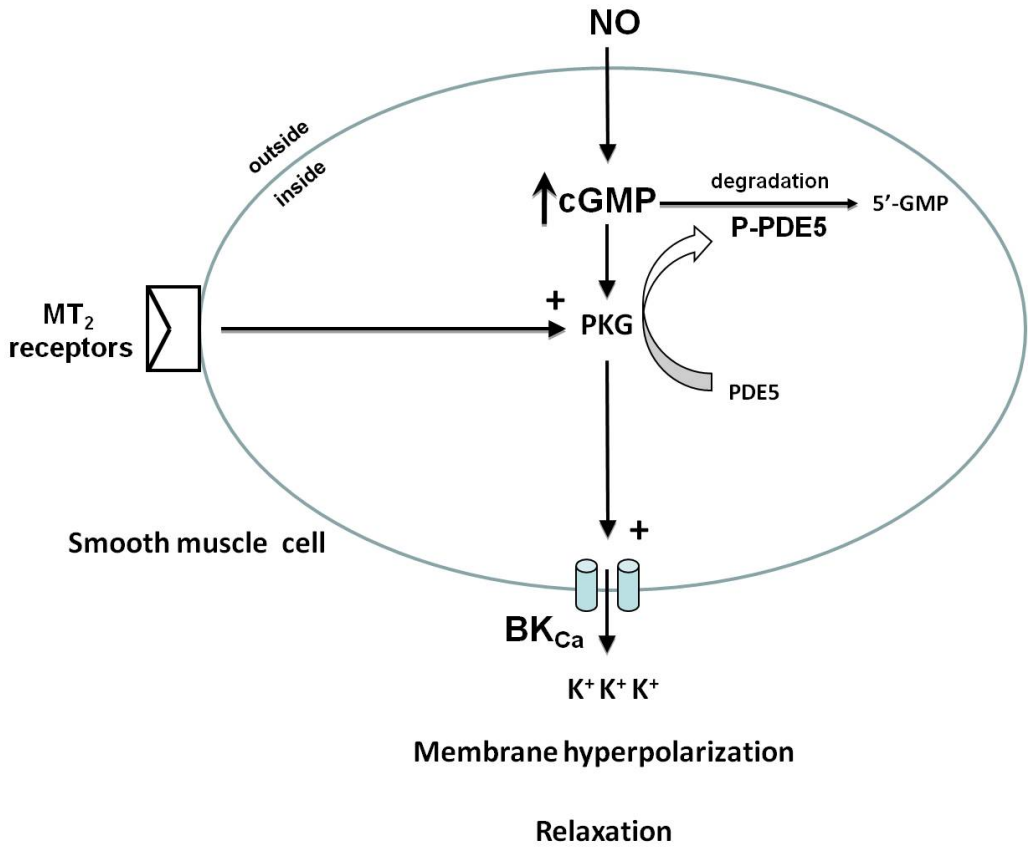
The data indicate that activation of MT_2-receptors increases PDE5 phosphorylation in coronary arteries, likely via activation of PKG1, which results in inhibition of NO-mediated increases in intracellular cyclic GMP and smooth muscle relaxation. This is the first report showing a direct role of increased PDE5 phosphorylation and activity in the inhibitory effect of melatonin on NO/cyclic GMP signaling in coronary arteries. One implication of these findings is that melatonin

may potentially reduce the efficacy of pharmacologic agents that act by releasing NO e.g. nitro vasodilators, by enhancing the PDE5-mediated degradation of cyclic GMP.

The large conductance, calcium-activated K channels (BK_{Ca}) are the predominant K channel expressed in coronary smooth muscle. BK_{Ca} is activated by both Ca^{2+} and voltage which favors it functioning as a dominant repolarizing current to act as a negative feedback control mechanism for contractile stimuli. Relaxation of vascular smooth muscle cells induced by endothelium-derived NO and exogenous NO-donors is predominantly mediated by activation of BK_{Ca} channels. NO-induced activation of BK_{Ca} channels leads to membrane hyperpolarization and a reduction in intracellular Ca^{2+} concentration and relaxation. In organ chamber studies, blockade of BK_{Ca} channels by iberiotoxin, a potent and selective BK_{Ca} channel blocker caused a significant rightward shift in the concentration-response curve for SNP supporting a crucial role for BK_{Ca} channels in NO-induced relaxation of porcine coronary arteries. It is know that, in functional vascular reactivity studies, at concentrations similar to that used in this study, melatonin causes melatonin-receptor dependent inhibition of BK_{Ca} channel activity in small and large diameter arteries. Therefore it is possible that the impairment of NO-induced relaxation of porcine coronary arteries by melatonin involves inhibition of NO-activated BK_{Ca} channel activity in porcine coronary smooth muscle cells.

The results of this study indicate that impairment of coronary artery relaxation in response to NO by melatonin involves MT_2-receptor coupled inhibition of NO-activated BK_{Ca} currents. In support of this conclusion, I determine that melatonin inhibits the BK_{Ca} currents evoked by NO, but not by 8-Br-cGMP in coronary smooth muscle cells and blockade of either MT_2 receptors or PDE5 abolished the inhibitory effect of melatonin on the NO-induced increase in BK_{Ca} current, suggesting a mechanistic coupling between MT_2-receptor activation and PDE5 activity. Moreover, inhibition of soluble guanylyl cyclase with ODQ abolished the NO-induced relaxation of coronary arteries; increase in intracellular cGMP levels, and increase in whole-cell BK_{Ca} currents in coronary smooth muscle cells suggesting that the cGMP-dependent activation of BK_{Ca} channels mediates NO-induced relaxation of coronary arteries. Taken together; the results suggest that melatonin

increases PDE5-mediated cGMP degradation by activating MT_2-receptors, resulting in inhibition of BK_{Ca} channel activation in response to NO and impaired NO-induced vasorelaxation.



Scheme 1. The proposed mechanism by which melatonin inhibits NO-induced relaxation of porcine coronary artery. Melatonin activates MT_2 receptors (a G-protein coupled receptor), present on vascular smooth muscle cells, stimulating the PKG-dependent phosphorylation, of PDE5, and thereby increasing enzyme catalytic activity several-fold. Activated PDE5 degrades active cGMP into inactive 5'-GMP resulting in decreased activation of BK_{Ca} channels and inhibition of NO-induced relaxations of coronary smooth muscle.

There are several future directions that can be built on the work of this project. A logical extension of all these studies would be to determine if melatonin exerts similar inhibitory effect in human blood vessels e.g. human radial arteries. Although, the data from this study strongly support a role for MT_2-receptors, we cannot rule out a possible role for MT_1-receptors in the observed effect of melatonin. I did not find the expression of MT_1-receptors in porcine coronary arteries, but MT_1-

receptors are expressed in human coronary arteries with unknown function. At present, there are no potent and selective MT_1-receptor antagonists available. However, if such pharmacologic tools become available it would be very interesting to determine the role of MT_1-receptors if any in the inhibitory effect of melatonin on NO-induced relaxation of human arteries.

Most of the available PDE activity assays involve the measurement of PDE activity in tissue or cell lysates and not in whole tissue systems. That the MT_2-receptor selective antagonist, 4P-PDOT, abolished the inhibitory effect of melatonin on the NO-induced increase in cGMP level, strongly supports the need to measure PDE activity in intact coronary arterial rings with functional MT_2-receptor signaling pathways. Thus, I have used PDE5 phosphorylation as a surrogate marker for the increased activity of PDE5 by melatonin. It would be interesting to determine the effect of melatonin of PDE5 activity directly, if assays supporting the measurement of PDE5 activity in intact tissue or cell system become available.

In the present study, I exclusively focused on elucidating the mechanism by which melatonin inhibits NO/cyclic GMP signaling at vascular smooth muscle level. However, my preliminary data suggest that melatonin also inhibits relaxation of coronary arteries induced by endothelium-dependent NO-donors. Therefore, investigating the potential effects of melatonin on endothelial NO biosynthetic pathway represent another challenging research avenue.

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