



## Research Paper

## Lectin-induced oxidative stress in human platelets

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## ABSTRACT

Previously we have shown that *wheat germ agglutinin* (WGA) and, with minor potency, *Phaseolus vulgaris agglutinin* (PHA), but not *lens culinaris agglutinin* (LCA), induce platelet aggregation, through the PLC $\gamma$ 2 activation by the concerted action of src/syk and PI3K/BTK pathways. In this study, we have investigated platelet oxidative stress induced by lectins. Several parameters indicative of oxidative stress, such as reactive oxygen species (ROS), superoxide anion, lipid peroxidation and the efficiency of the aerobic metabolism, have been measured. It was found that ROS, superoxide anion formation and lipid peroxidation are significantly increased upon platelet treatment with WGA and PHA while LCA is ineffective. WGA is always more effective than PHA in all experimental conditions tested. In addition, the involvement of NADPH oxidase 1, syk and PI3K in oxidative stress induced by WGA and PHA has been shown. Concerning the lectins effect on aerobic metabolism, WGA and PHA, but not LCA, act as uncoupling agents, determining an increase of oxygen consumption and a decrease of ATP synthesis, with a consequent decrease of P/O value. These results are confirmed by the impairment of platelets proton gradient formation, evaluated by membrane potential, in platelets treated with WGA and PHA. In conclusion lectins, especially WGA, induce oxidative stress in platelets and decrease energy availability through modifications of membrane structure leading to the inefficiency of the aerobic machinery that steers platelets toward death as suggested by the decreased metabolic activity of platelets and the increased lactic dehydrogenase release.

## 1. Introduction

Reactive oxygen species (ROS) include oxygen ions, free radicals and peroxides. Most intracellular ROS are derived from superoxide anion formed principally by mitochondria [1]. Among ROS superoxide anion and hydrogen peroxide are considered critical messengers for initiating changes in cellular signalling events [2,3]. Moreover, ROS can react with lipids, proteins and DNA causing irreversible damage in their structure and function [4–7]. Therefore, ROS are important mediators of cellular and tissue injury implicated in a causative factor in several diseases such as atherosclerosis [8,9] diabetes [10] and neurodegeneration [11], as well as in aging [8,12]. There is increasing evidence demonstrating that ROS regulate signalling pathways involved in physiological and pathological processes [3,13]. Platelets are activated upon exposure to ROS, including those derived from polymorphonuclear leukocytes and red blood cells [14]. Platelets produce significant levels of superoxide anion or hydrogen peroxide upon stimulation by agonists [15–19]. In addition, in the presence of high

intracellular ROS concentration, platelets demonstrate increased activation by agonists [20].

Lectins are plant-derived proteins [21], ubiquitous in nature, that recognise and bind specific carbohydrate of cell surface or glycocalyx causing clusterization of membrane glycoreceptor, transmembrane signalling and cell aggregation similar to physiological agonists of platelets [22,23]. *Wheat germ agglutinin* (WGA) is a cereal lectin specific for two types of N-acetylated sugar, N-acetyl-D-glucosamine and N-acetyl-D-neuraminic acid [24]. *Phaseolus vulgaris agglutinin* (PHA) has a sugar specificity for glucosaminyl and mannopyranosyl residue [25] and *lens culinaris agglutinin* (LCA) is a legume lectin that binds specifically to mannose [26] and fucose [27]. Previously [28], we have shown that WGA and PHA, but not LCA, are able to stimulate platelet aggregation through the same mechanism, being WGA more active than PHA. Both lectins stimulate the signalling pathways src/syk and PI3K/AKT/BTK and induce the PLC $\gamma$ 2 phosphorylation/activation with the consequent rapid increase of intracellular Ca<sup>2+</sup> level [28]. Recently, we have shown that PHA induces nitric oxide elevation in platelets,

**Abbreviations:** LCA, lens culinaris agglutinin; PHA, Phaseolus vulgaris agglutinin; Nox, NADPH oxidase; ROS, reactive oxygen species; WGA, wheat germ agglutinin

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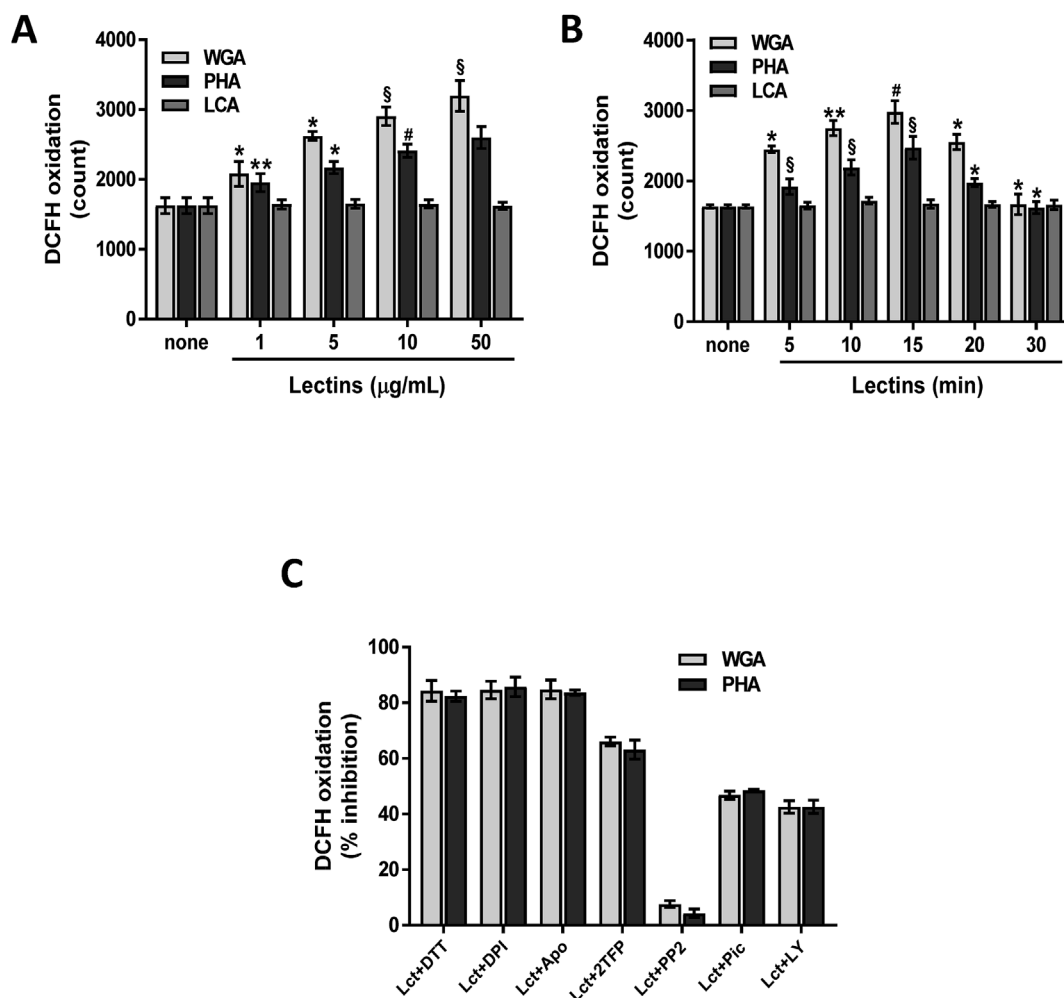
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**Fig. 1. ROS formation induced by lectins.** Washed platelets ( $1.0 \times 10^8$ /mL), loaded with  $10 \mu\text{M}$  DCFH-DA and preincubated with saline, were stimulated for 15 min with lectins as indicated (panels A) or with  $10 \mu\text{g/mL}$  lectins for the indicated times (panel B). In panel C, washed platelets ( $1.0 \times 10^8$  platelets/mL), loaded with  $10 \mu\text{M}$  DCFH-DA, were preincubated 10 min with saline,  $100 \mu\text{M}$  DTT,  $50 \mu\text{M}$  DPI,  $100 \mu\text{M}$  apocynin (Apo),  $10 \mu\text{M}$  2-TFP (2TFF),  $10 \mu\text{M}$  PP2,  $30 \mu\text{M}$  Piceatannol (Pic) or  $20 \mu\text{M}$  LY294002 (LY) and then stimulated for 15 min with  $10 \mu\text{g/mL}$  lectins. At the end of incubation, samples were immediately analyzed by flow cytometry. Data are the mean  $\pm$  SD of at least six experiments carried out in duplicate. Mann-Whitney  $U$  test: \* $P < 0,0001$ , \*\* $P < 0,0005$ ,  $\S P < 0,001$ , # $P < 0,01$ .

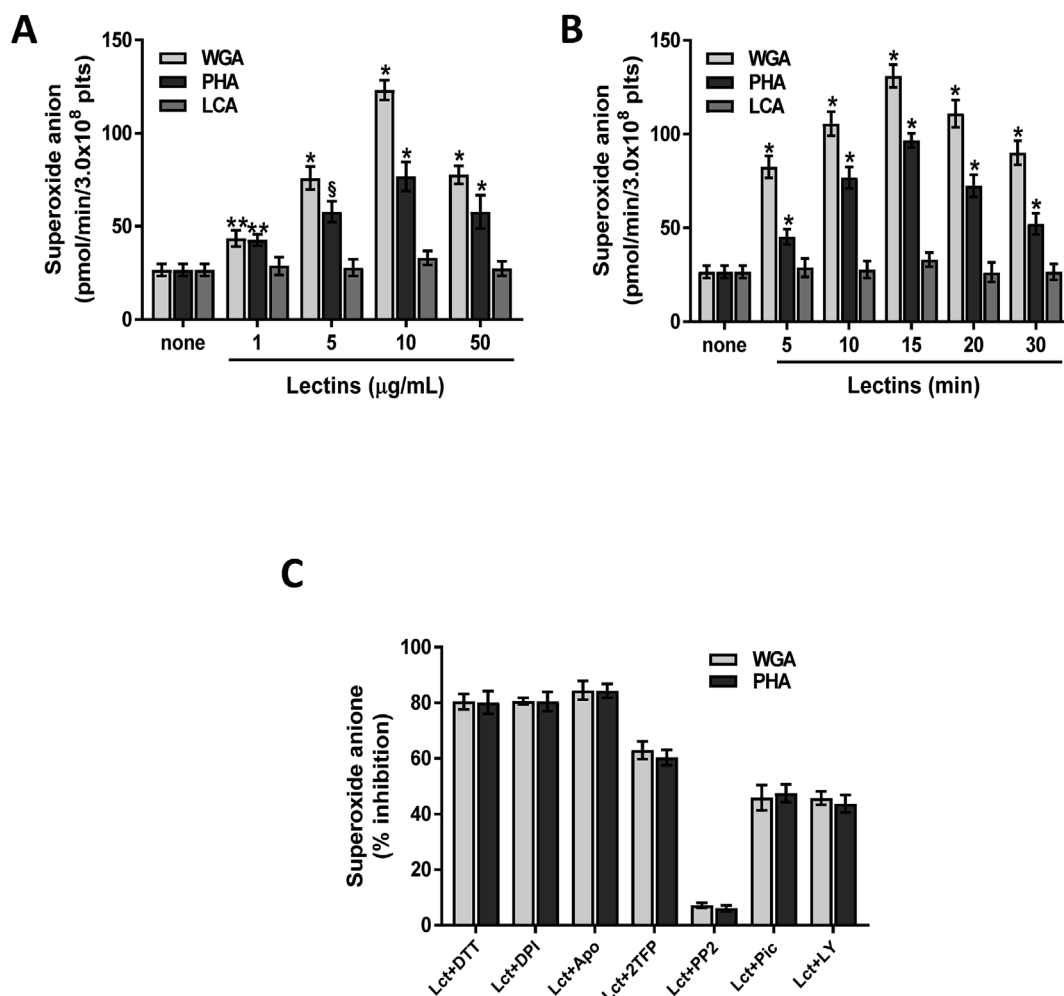
stimulating endothelial nitric oxide synthase phosphorylation/activation, mainly through the activation of the  $\text{Ca}^{2+}$ /Calmodulin dependent kinase kinase  $\beta$  (CaMKK $\beta$ )/AMPK $\alpha$  pathway [29]. On the contrary WGA does not stimulate nitric oxide elevation in human platelets [29]. In the present paper, the effect of WGA, PHA and LCA on several parameters indicative of cell oxidative status in platelets, such as ROS, superoxide anion, lipid peroxidation and GSH content, have been tested. We have found that all these parameters are increased in platelets stimulated by WGA and PHA, but not by LCA. Moreover, evaluating the efficiency of the aerobic metabolism in WGA or PHA treated platelets, a decrease of P/O value was observed, due to the increased oxygen consumption and to the decreased ATP formation, suggesting that lectins could be considered uncoupling agents. Finally, the involvement of NADPH oxidase (Nox) and syk/PI3K in lectin-mediated ROS elevation was shown. In agreement with previous data [28], WGA, that behaves as a physiological agonist, is more powerful than PHA towards all the tested parameters, while LCA is ineffective.

## 2. Material and methods

### 2.1. Materials

Apocynin, apyrase, bovin serum albumin, butylated

hydroxytoluene, cytochrome C, dichlorofluorescein diacetate (DCFH-DA), diphenyleneiodonium (DPI), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), L-lactic dehydrogenase (EC 1.1.1.27), leupeptin,  $\text{NAD}^+$ , NADH, PGE1, piceatannol, phenylmethylsulfonyl fluoride (PMSF), PP2 analogue (PP2), protease inhibitor cocktail (Cat. N° P8340), superoxide dismutase (SOD), thiobarbituric acid (TBA), digonin, pyruvate, malate, succinate, ouabain, ampicillin, di-adenosine-5'penta-phosphate, rotenone, antimycin A, ADP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and all chemicals were from Sigma-Aldrich, USA. Lectins (WGA, PHA, LCA) and LY294002 were purchased from Merck Millipore, Germany. 2(trifluoromethyl) phenothiazine (2TFF), specific inhibitor of Nox1 [30], was a gift of Prof. Bruno Tasso Dept. Pharmacy, Genoa University, Genoa. Apocynin, DPI, DTT, LY294002, piceatannol, PP2, 2TFF and FCCP, were diluted in saline from a stock DMSO solution immediately before each experiment. MitoProbe™ Tetramethylrhodamine Methyl Ester (TMRM) from ThermoFisher Scientific was a gift from Dr. Paolo Degan, UO Mutagenesi e Prevenzione Oncologica, IRCCS Ospedale Policlinico San Martino, Genoa. ATP bioluminescence assay kit CLSII and ATP standard solution were from Roche, Switzerland.



**Fig. 2. Superoxide anion formation induced by lectins.** Washed platelets ( $5.0 \times 10^8$ /mL), preincubated with saline in the presence of 100  $\mu$ M cytochrome C and 300 U SOD, if present, were stimulated for 15 min with lectins as indicated (panels A) or with 10  $\mu$ g/mL lectins for the indicated times (panel B). In panel C, washed platelets ( $5.0 \times 10^8$  platelets/mL) were preincubated 10 min with saline, 100  $\mu$ M DTT, 50  $\mu$ M DPI, 100  $\mu$ M apocynin (Apo), 10  $\mu$ M 2-TFP (2TFP), 10  $\mu$ M PP2, 30  $\mu$ M Piceatannol (Pic) or 20  $\mu$ M LY294002 (LY) in the presence of 100  $\mu$ M cytochrome C and 300 U SOD, if present and then stimulated for 15 min with 10  $\mu$ g/mL lectins. The cytochrome C reduction was measured by spectrophotometry as detailed in Methods. Data are the mean  $\pm$  SD of at least six experiments carried out in duplicate. Mann-Whitney *U* test: \**P* < 0,0001, \*\**P* < 0,0005, §*P* < 0,001.

## 2.2. Methods

### 2.2.1. Blood collection and preparative procedures

Freshly drawn venous blood from healthy volunteers of the “Centro Trasfusionale, Ospedale San Martino” in Genoa was collected into 130 mM aqueous trisodium citrate anticoagulant solution (9:1). The donors claimed to have not taken drugs known to interfere with platelet function during two weeks prior to blood collection and gave their informed consent. Washed platelets were prepared centrifuging whole blood at  $100 \times g$  for 25 min. To the obtained platelet-rich plasma (PRP) 4 mU/mL apyrase and 4  $\mu$ M PGE1 were added. PRP was then centrifuged at  $1100 \times g$  for 15 min. Pellet was washed once with pH 5.2 ACD solution (75 mM trisodium citrate, 42 mM citric acid and 136 mM glucose), centrifuged at  $1100 \times g$  for 15 min and then resuspended in  $\text{Ca}^{2+}$ -free HEPES buffer containing 145 mM NaCl, 5 mM KCl, 1 mM  $\text{MgSO}_4$ , 10 mM glucose, 10 mM Hepes (pH 7.4).

### 2.2.2. ROS assay

ROS were measured as previously described [15,16] with slight modifications. Briefly, washed platelets ( $1.0 \times 10^8$ /mL) were preincubated with saline or inhibitors for 15 min at 37 °C in the presence of 10  $\mu$ M DCFH/DA and then incubated with lectins. At the end of incubation, samples were put in ice and the stable and highly fluorescent

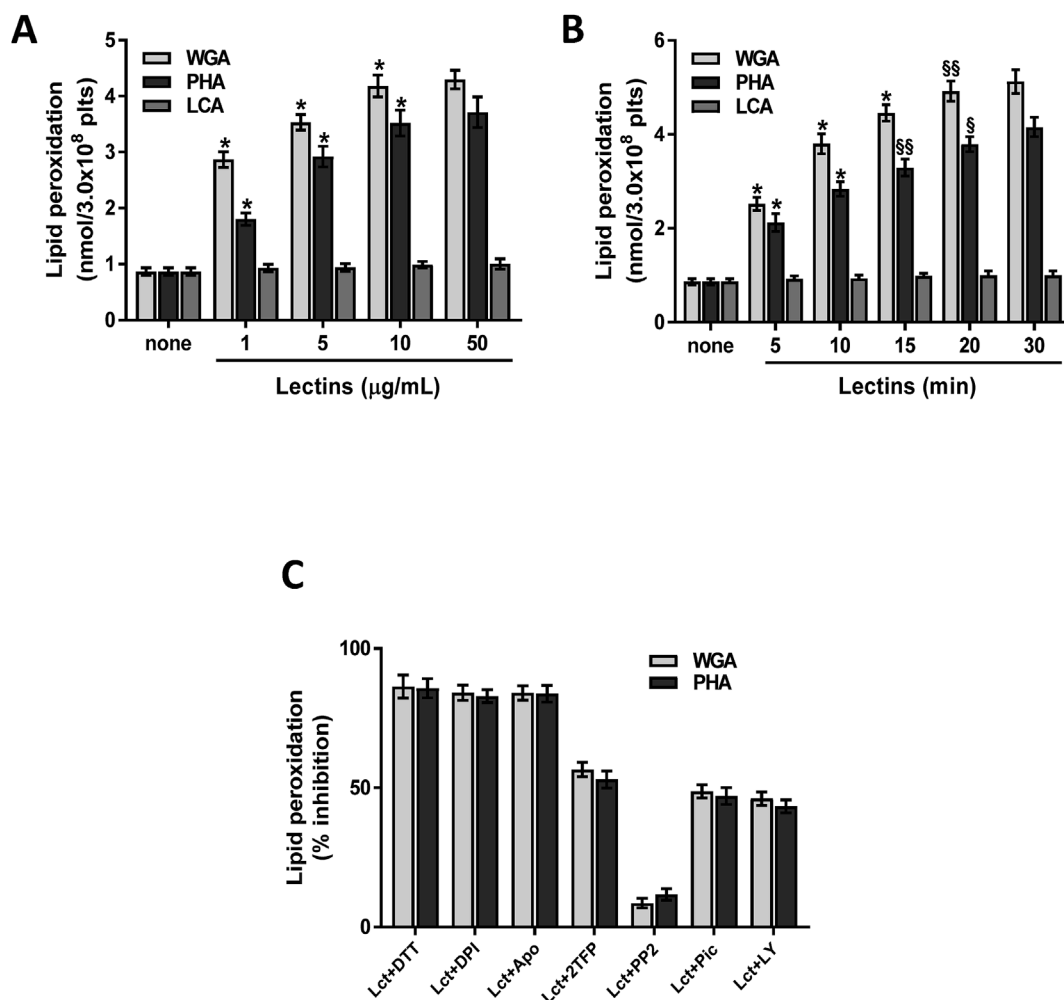
2,7-dichlorofluorescein (DCF), obtained by the ROS-induced DCFH oxidation, was immediately measured by flow cytometry in a Merck Millipore Bioscience Guava EasyCyte flow cytometer.

### 2.2.3. Superoxide anion assay

The production of superoxide anion was measured by mean of the difference between total and SOD-inhibitable cytochrome C reduction as described [15,31] with light modifications. Washed platelets ( $5.0 \times 10^8$ /mL), preincubated with saline or inhibitors for 10 min at 37 °C in the presence of 100  $\mu$ M cytochrome C and 300 U SOD, if present, were challenged with lectins. Incubation was stopped by putting samples in ice. Samples were sedimented by centrifugation at 12000g for 8 min and reduced cytochrome C was measured in the supernatant by spectrophotometry at 550 nm, in a Beckman DU530 spectrophotometer, with molar extinction coefficient of  $21,100 \text{ M}^{-1} \text{ cm}^{-1}$ .

### 2.2.4. Lipid peroxidation measurement

Lipid peroxidation was quantified by measuring thiobarbituric acid reactive substances (TBARS) as described [32] with slight modifications. Washed platelets ( $5.0 \times 10^8$ /mL), preincubated with saline or inhibitors for 10 min at 37 °C in the presence of butylated hydroxytoluene, were stimulated with lectins. Incubation was stopped by



**Fig. 3. Lipid peroxidation induced by lectins.** Washed platelets ( $5.0 \times 10^8$ /mL), preincubated with saline were stimulated for 15 min with lectins as indicated (panels A) or with 10 µg/mL lectins for the indicated times (panel B). In panel C, washed platelets ( $5.0 \times 10^8$  platelets/mL) were preincubated 10 min with saline, 100 µM DTT, 50 µM DPI, 100 µM apocynin (Apo), 10 µM 2-TFP (2TFP), 10 µM PP2, 30 µM Piceatannol (Pic) or 20 µM LY294002 (LY) and then stimulated for 15 min with 10 µg/mL lectins. Lipid peroxidation was quantified by measuring thiobarbituric acid reactive substances (TBARS) as detailed in Methods. Data are the mean  $\pm$  SD of at least six experiments carried out in duplicate. Mann-Whitney *U* test: \**P* < 0,0001, §*P* < 0,001, §§*P* < 0,005.

cooling the samples in an ice bath in the presence of an equal volume of 20% trichloroacetic acid in 0.6 N HCl. One volume of supernatant obtained after 12000g for 5 min centrifugation was mixed with 0.2 volume of 0.12 M TBA in 0.26 M Tris (pH 7,0) and incubated for 30 min at 70 °C. The TBARS produced were assayed spectrophotometrically at 532 nm, in a Beckman DU530 spectrophotometer, with molar extinction coefficient of  $156,000 \text{ M}^{-1} \text{ cm}^{-1}$ .

#### 2.2.5. GSH and membrane SH-group assay

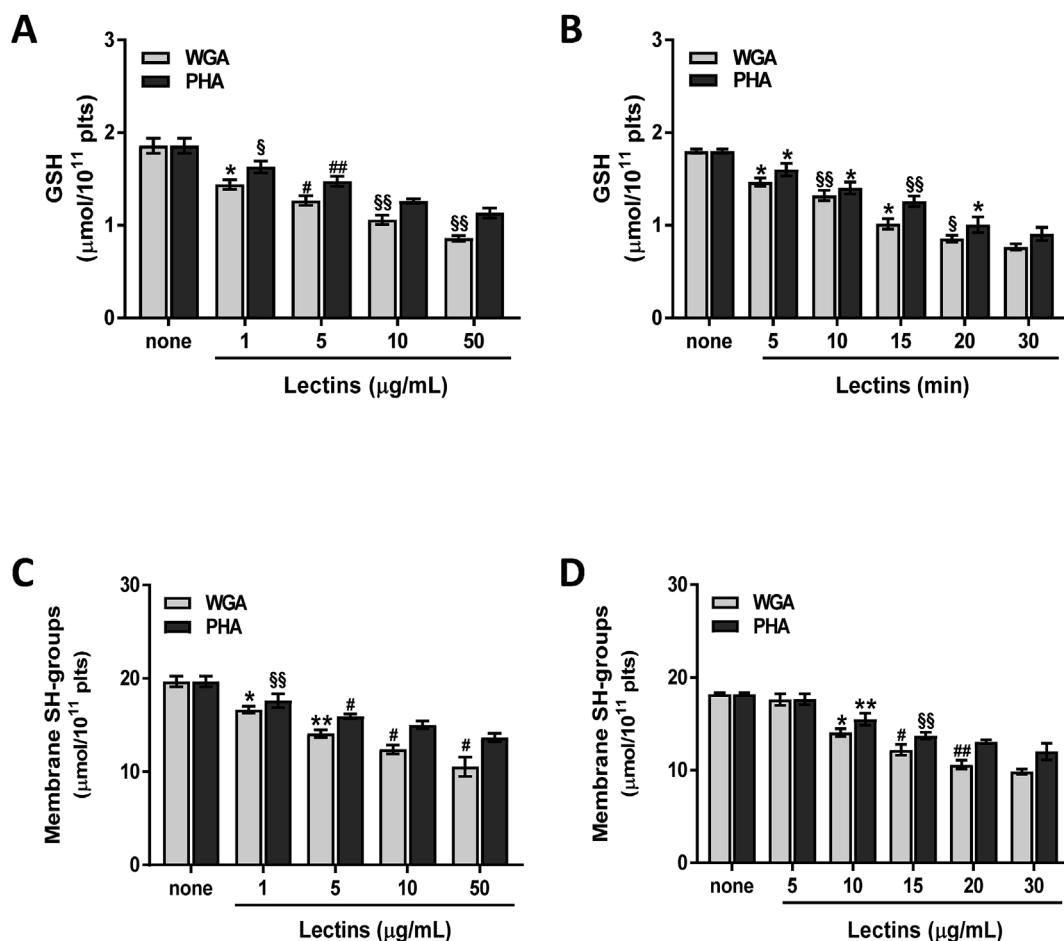
The GSH content was quantified by properly modified Tietze method [33]. Briefly, washed platelets ( $4.0 \times 10^9$ /mL) preincubated with saline for 10 min at 37 °C, were stimulated with lectins. Incubation was stopped by adding 0.2 M metaphosphoric acid and then the samples were centrifuged. The GSH content was determined in the supernatant that was immediately mixed with 0.5 mM DTNB and 0.3 M  $\text{Na}_2\text{HPO}_4$ . The membrane SH-groups were measured in the pellet obtained by the centrifugation, first resuspended in 3% SDS and then mixed with 0.5 mM DTNB and 0.3 M  $\text{Na}_2\text{HPO}_4$ . The GSH and the membrane SH-groups were both quantified by spectrophotometry at 412 nm, in a Beckman DU530 spectrophotometer, with molar extinction coefficient of  $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ .

#### 2.2.6. NADPH oxidase activity assay

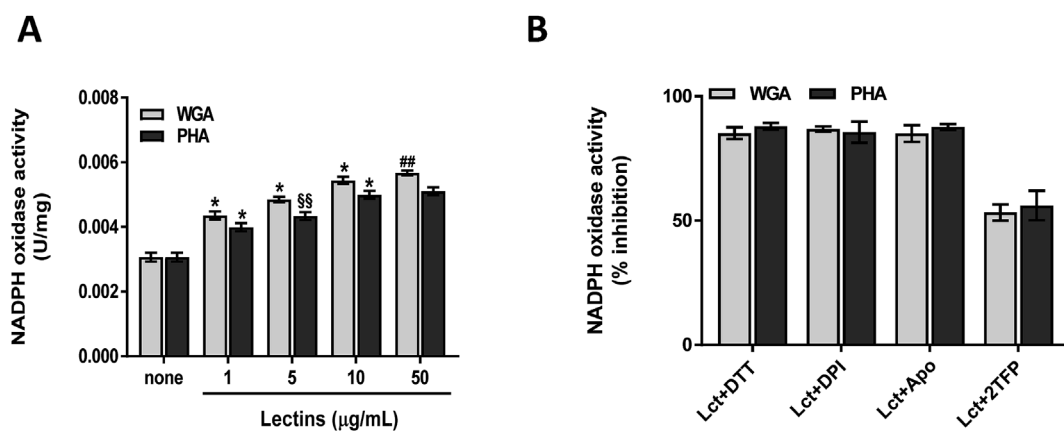
The enzymatic activity of NADPH oxidase was assessed spectrophotometrically in platelet homogenates by measuring the reduction of cytochrome C at 550 nm. Briefly, washed platelets ( $1.0 \times 10^9$ /mL), added to 10 µg/mL leupeptin, 1 mM PMSF, 100 µM DTT and 1/100 dilution protease inhibitor cocktail, were sonicated twice for 15 s and then centrifuged at  $14000 \times g$  for 10 min. Aliquots of the obtained supernatant, preincubated with saline or inhibitors for 10 min at 37 °C, were treated with lectins. Incubation was stopped by cooling samples in ice and NADPH oxidase activity was assayed as reported [15]. Protein concentration was measured by Lowry method with bovine serum albumin as standard protein [34].

#### 2.2.7. Oximetric analysis

The oxygen consumption was measured at 37 °C with an amperometric  $\text{O}_2$  electrode in a closed chamber (Unisense, DK). Washed platelets ( $1.0 \times 10^9$ /mL) were preincubated with saline and then treated at 37 °C with lectins or 10 µM FCCP. Incubation was stopped by putting samples in ice. From each sample 10 µg of total proteins, determined by Bradford assay [35], were resuspended in a medium containing 137 mM NaCl, 5 mM  $\text{KH}_2\text{PO}_4$ , 5 mM KCl, 0.5 mM EDTA, 3 mM  $\text{MgCl}_2$  and 25 mM Tris, pH 7.4 and permeabilized with 0.03% digitonin for 10 min. To stimulate the Complexes I, III and IV, 10 mM pyruvate and



**Fig. 4.** GSH and membrane SH-group levels in human platelets treated with lectins. Washed platelets ( $4.0 \times 10^9$ /mL), preincubated with saline, were stimulated for 15 min with lectins as indicated (panels A) or with 10 µg/mL lectins for the indicated times (panel B). At the end of incubation samples were precipitated by metaphosphoric acid and GSH or membrane SH-groups quantified as detailed in Methods. Data are the mean  $\pm$  SD of at least six experiments carried out in duplicate. Mann-Whitney *U* test: \**P* < 0,0001, \*\**P* < 0,0005, §*P* < 0,001, §§*P* < 0,005, #*P* < 0,01, ##*P* < 0,05.



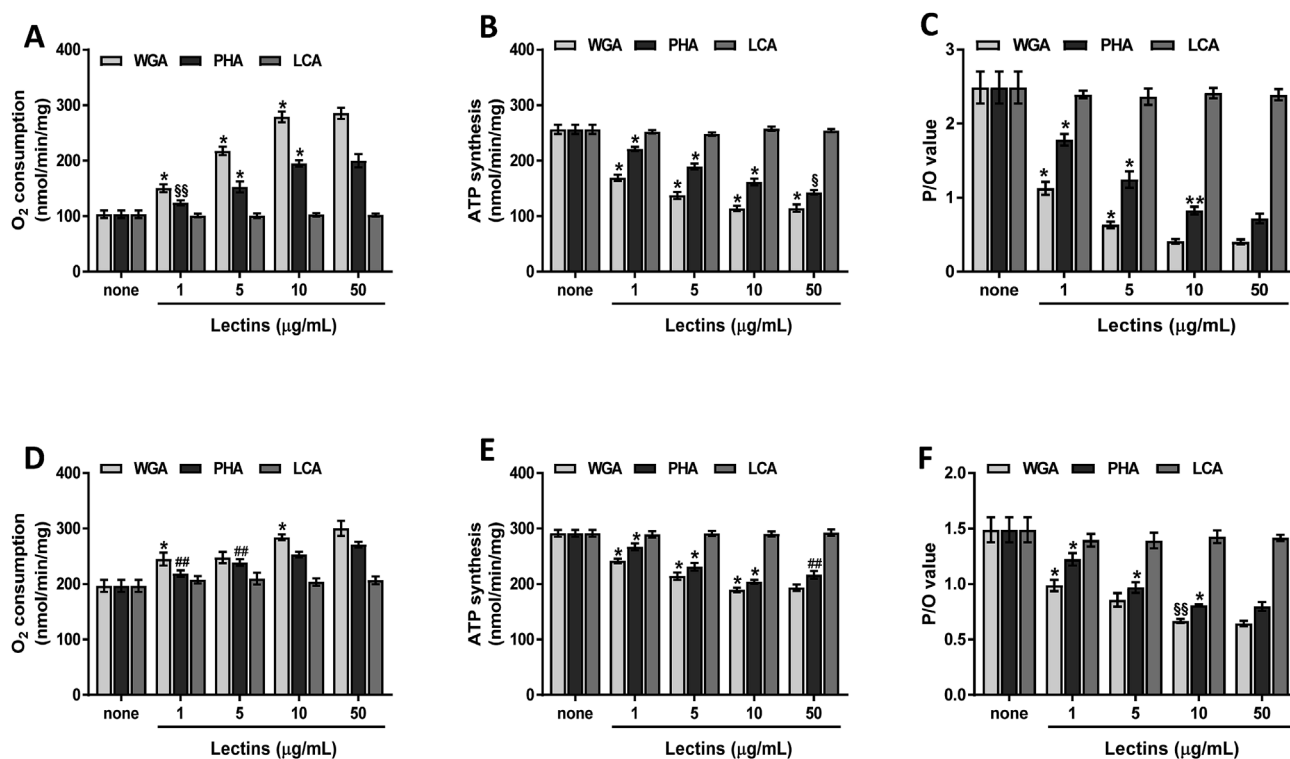
**Fig. 5.** NADPH oxidase activity in lectin treated platelets. Washed platelets ( $2.0 \times 10^9$ /mL), preincubated with saline were treated with lectins as indicated (panel A) or preincubated with 100 µM DTT, 50 µM DPI, 100 µM apocynin (Apo), 10 µM 2-TFP (2TFP) and then stimulated with 10 µg/mL lectin (panel B). At the end of incubation samples were sonicated and centrifuged. NADPH oxidase activity was assayed in the supernatant as detailed in Methods. Data are the mean  $\pm$  SD of at least six experiments carried out in duplicate. Mann-Whitney *U* test (panel A): \**P* < 0,0001, §§*P* < 0,005, ##*P* < 0,05. Student's *t*-test (panel B): §*P* < 0.0001 vs none; \**P* < 0.0001 vs Lct.

5 mM malate in the presence of 0.2 mM ADP were added, while 20 mM succinate in the presence of 0.2 mM ADP were used to activate the Complexes II, III and IV [36]. In others experiments, washed platelets ( $1.0 \times 10^9$ /mL) were preincubated for 10 min at 37 °C with saline or inhibitors and then stimulated by lectins or FCCP directly in the closed

chamber.

### 2.2.8. Evaluation of Fo-F1 ATP synthase activity

Washed platelets ( $1.0 \times 10^9$ /mL) were preincubated with saline or inhibitors for 10 min at 37 °C and stimulated with lectins. Incubation



**Fig. 6. Aerobic metabolism measured in the presence of lectins.** Washed platelets ( $1.0 \times 10^9$ /mL) preincubated with saline, were stimulated for 1 min at 37 °C with lectins. Panels A–C show the oxygen consumption, the aerobic ATP synthesis and the P/O ratio, respectively, in the presence of pyruvate + malate. Panels D–F show data obtained in the presence of succinate. Data are the mean  $\pm$  SD of at least six experiments. Oxygen consumption is expressed as nmol of oxygen consumed per minute and per mg of total protein (nmol O<sub>2</sub>/min/mg). ATP synthesis is expressed as nmol of synthesized ATP per minute and per 1 mg of total protein (nmol ATP/min/mg). Mann-Whitney *U* test: \**P* < 0,0001, \*\**P* < 0,0005, §*P* < 0,001, §§*P* < 0,005, ##*P* < 0,05.

was blocked cooling samples in ice, then 10 µg of total protein, determined by Bradford assay [35], of each sample were added to the incubation medium (0.1 mL final volume), containing 10 mM Tris (pH 7.4), 50 mM KCl, 1 mM EGTA, 2 mM EDTA, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 0.6 mM ouabain, 0.040 mg/mL ampicillin, 0.2 mM di-adenosine-5′penta-phosphate and the metabolic substrates 10 mM pyruvate + 5 mM malate or 20 mM succinate. Platelets were warmed for 10 min at 37 °C, then ATP synthesis was induced by the addition of 0.1 mM ADP. The ATP synthesis was measured using the luciferin/luciferase ATP bioluminescence assay kit CLSII, on a Promega GloMax® 20/20 Luminometer. ATP standard solutions were used in the range  $10^{-10}$  -  $10^{-7}$  M for calibration [36]. In others experiments, 10 µg of total proteins were preincubated with saline, inhibitors or FCCP and then treated with lectins as above detailed.

### 2.2.9. P/O ratio calculation

The P/O value is the ratio of the nmol of ATP produced to the nmol of oxygen consumed. In coupled conditions, when the oxygen consumption is completely associated to the ATP synthesis, the P/O ratio is about 2.5 or 1.5 in the presence of pyruvate + malate or succinate, respectively [37]. In the uncoupled status, the decrease of this value is in correlation with the degree of the oxidative phosphorylation inefficiency.

### 2.2.10. Membrane potential determination

Washed platelets ( $1.0 \times 10^9$ /mL) were preincubated with saline and then stimulated with lectins. Incubation was stopped by putting samples in ice. Then samples were loaded with 50 nM TMRM for 10 min at 37 °C and the membrane potential immediately measured by flow cytometry in a Merck Millipore Bioscience Guava EasyCyte flow cytometer.

### 2.2.11. Platelet viability assays

To check up platelet viability upon lectins treatment the efficiency of the glycolytic pathway was measured by the production of L-lactate according to Hohorst [38] and the membrane damage was verified by the L-lactic dehydrogenase released from platelets according to Vasault [39].

### 2.2.12. Statistical analysis

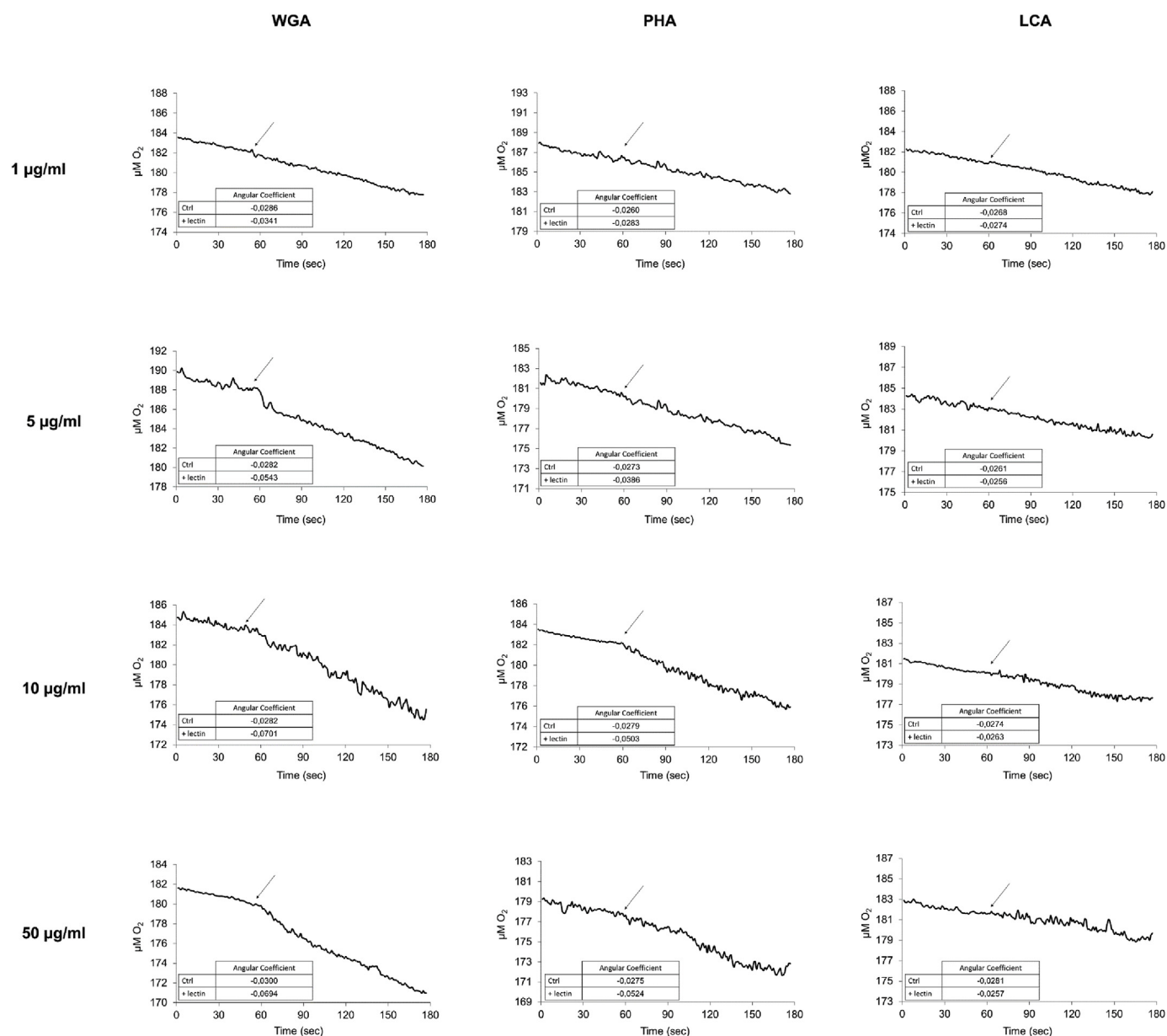
Data are mean  $\pm$  SD of at least five independent experiments, each performed in duplicate. Statistical comparisons between two groups were performed through the unpaired Student's *t*-test. To compare multiple groups Mann-Whitney *U* test was used. Statistical significance was defined as *p* < 0.05.

## 3. Results

### 3.1. ROS production in platelets treated with lectins

In agreement with other agonists such as thrombin, PMA and arachidonic acid, WGA and PHA produce DCFH conversion in DCF, being the lectins effect dose- and time-dependent with a peak at 50 µg/mL (Fig. 1A) and at 15 min (Fig. 1B), respectively. WGA always more effective than PHA has been found, while no effect of LCA was shown. In Fig. 1C several compounds able to inhibit oxidative stress induced by WGA or PHA have been tested. It has been found that the protein disulphide reducing agent DTT, DPI, considered a non-selective inhibitor of Nox and the antioxidant agent apocynin, a non-selective Nox inhibitor, inhibit DCFH oxidation more than 80%. An inhibition of about 60% has been measured in platelets pre-treated with the specific inhibitor of Nox1, 2TFP. Since ROS regulate specific pathways involved in platelet activation, we wanted to clarify which enzyme could contribute to ROS increase in platelets stimulated by lectins. Lectins induced DCFH





**Fig. 7.** Time course of oxygen consumption induced by lectins. Panels show the amperometric traces, measured directly in the closed chamber with the  $O_2$  electrode, of washed platelets ( $1.0 \times 10^9/\text{mL}$ ) stimulated by varying concentrations of lectins, in the presence of pyruvate + malate. Tracings are representative of at least six independent experiments and data are expressed as micromolar concentration of consumed oxygen ( $\mu\text{M } O_2$ ).

oxidation is not affected by the src kinase inhibitor PP2 and it is inhibited of about 40–50% by the PI3K inhibitor LY294002 and by the syk inhibitor piceatannol. Moreover, rotenone or antimycin A, inhibitors of respiratory chain complexes I and III, are ineffective (data not shown).

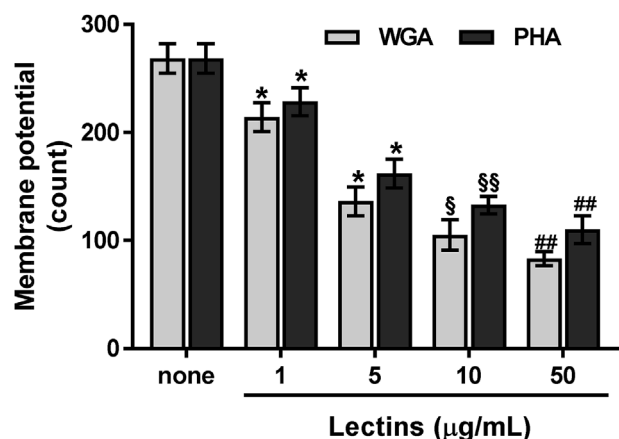
### 3.2. Superoxide anion formation in platelets treated with lectins

Platelets have reductive property toward cytochrome C and the aliquot of reduced cytochrome C which is inhibitable by SOD corresponds to the superoxide anion concentration. The basal amount of this oxygen radical produced in resting platelets significantly increases after treatment with WGA and PHA but not with LCA. The effect is dose-dependent peaking at  $10 \mu\text{g/mL}$  (Fig. 2A) and time-dependent reaching the maximum after 15 min of incubation at  $37^\circ\text{C}$  (Fig. 2B) for both lectins. WGA is more powerful than PHA in all tested conditions. Moreover, DTT, DPI and apocynin produce a strong inhibition on

superoxide anion formation ranging from 80 to 90% (Fig. 2C) and 2TFP an inhibition of about 60%. In agreement with data on DCFH oxidation (Fig. 1C) PP2 does not inhibit superoxide anion formation while LY294002 and piceatannol produce a quite similar inhibiting effect of about 40–50% (Fig. 2C).

### 3.3. Lipid peroxidation induced by lectins

Lipid peroxidation is a consequence of the increased ROS and superoxide anion formation. Thus, the lipid peroxidation induced by WGA and PHA is significantly increased. The lectins effect is dose- (Fig. 3A) and time-dependent (Fig. 3B), being WGA more effective than PHA in all conditions. As shown in Fig. 3C, the effect of DTT, DPI, apocynin, 2TFP, and PP2, piceatannol or LY294002 is like that measured on DCFH oxidation (Fig. 1C) or superoxide anion formation (Fig. 2C).



**Fig. 8.** Membrane potential in human platelets treated by lectins. Washed platelets ( $1.0 \times 10^9$ /mL), preincubated with saline, were treated with lectins as indicated. At the end of incubation samples were mixed with 50 nM TMRM and fluorescence quantified by flow cytometry as reported in Methods. Data are the mean  $\pm$  SD of at least six experiments carried out in duplicate. Mann-Whitney U test: \*P < 0,0001, §§P < 0,005, ##P < 0,05.

### 3.4. The WGA or PHA effect on GSH and membrane SH-groups

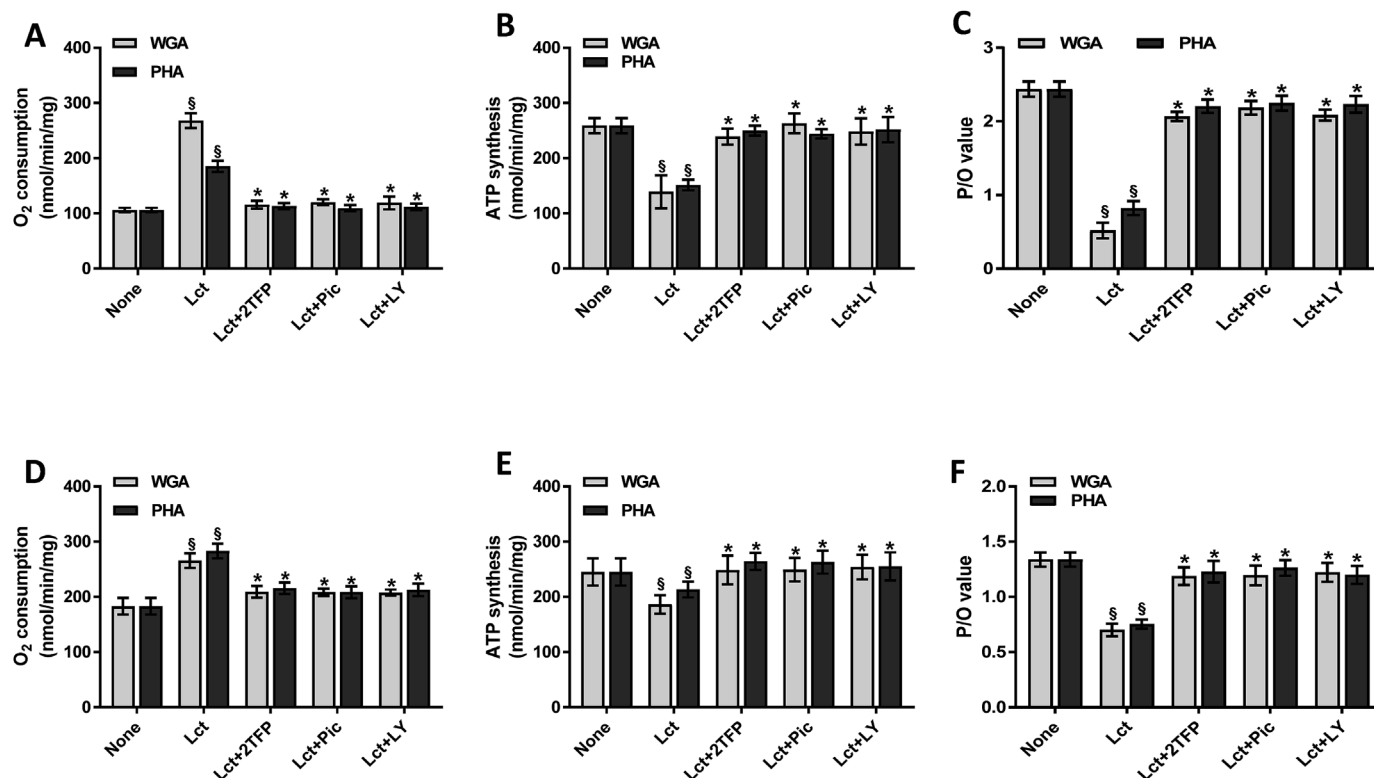
As shown in Fig. 4, in platelets treated with WGA and PHA the GSH content and the membrane SH-groups are decreased. The lectins effect is dose- (Fig. 4A,C) and time- (Fig. 4B,D) dependent, being higher for WGA than PHA at all tested concentrations. LCA does not produce effect (data not shown).

### 3.5. The WGA or PHA effect on NADPH oxidase activity

Data previously reported (Figs. 1C, 2C and 3C) suggest that Nox could be greatly involved in ROS elevation induced by WGA or PHA. Thus, the lectins effect on Nox activity was measured. It was found that WGA and PHA stimulate Nox, being the former more active than the latter (Fig. 5A). Moreover, in the presence of lectins DTT, DPI and apocynin produce a strong inhibition (about 80%) on Nox activity while, as expected, a specific Nox1 inhibitor 2TFP, reduces the enzymatic activity of about 60% (Fig. 5B).

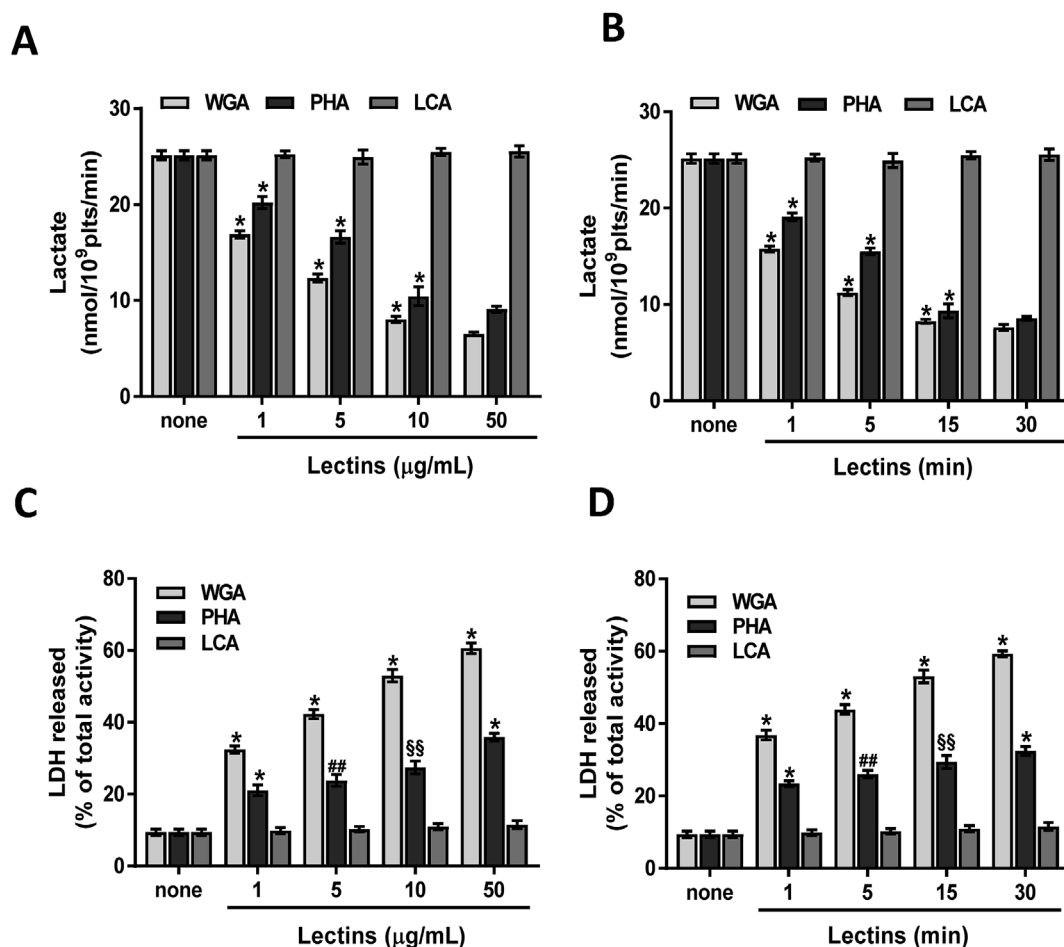
### 3.6. Effect of lectins on aerobic metabolism

Since oxidative stress could be also dependent on alteration of aerobic metabolism, in lectins-stimulated platelets we have measured the oxygen consumption and the ATP synthesis. In Fig. 6A,D, is shown that WGA and PHA increase oxygen consumption in a dose-dependent manner, being the first still more effective than the latter, both in the presence of pyruvate + malate or succinate. On the contrary, WGA or PHA have a negative effect on the aerobic ATP synthesis (Fig. 6B,E) in both metabolic conditions. In agreement with the other parameters tested, the oxygen consumption and ATP formation, in platelets treated with LCA does not change respect to the control. The ratio between the ATP synthesis and the oxygen consumption (P/O value), greatly decreases at 1 µg/mL and reaches the minimum at 10 µg/mL WGA or 50 µg/mL PHA (Fig. 6C, F). The same results have been obtained after 1 or 5 min of lectins incubation with human platelets (data not shown). The opposite effect of WGA and PHA on oxygen consumption and ATP synthesis suggests that these lectins behave as uncoupling agents. In fact, FCCP, a known uncoupling compound, produces the same effect as shown in Fig. 1 Supplementary C. In another set of experiments, lectins have been added to platelets directly in the closed chamber during the



**Fig. 9.** Effect of some NADPH oxidase inhibitors on the aerobic metabolism modified by lectins. Washed platelets ( $1.0 \times 10^8$ /mL), preincubated with saline or 10 µM 2-TFP (2TFP), 10 µM LY294002 (LY) or 30 µM piceatannol (Pic), were stimulated for 1 min with 10 µg/mL lectins. In Panels A–B the oxygen consumption and the aerobic ATP synthesis have been measured in the presence of pyruvate + malate and in panels D–E in the presence of succinate. In panels C–F the P/O ratios are reported. Data are the mean  $\pm$  SD of at least six experiments. Student's t-test: §P < 0.0001 vs none; \*P < 0.0001 vs Lct.





**Fig. 10.** Effect of lectins on platelet viability. Washed platelets ( $1.0 \times 10^9$ /mL), preincubated with saline, were stimulated for 15 min with lectins as indicated (panels A) or with 10  $\mu$ g/mL lectins for the indicated times (panel B). L-Lactate and L-lactic dehydrogenase were assayed as detailed in Methods. Data are the mean  $\pm$  SD of at least six experiments carried out in duplicate. Mann-Whitney *U* test: \**P* < 0,0001, §§*P* < 0,005, ##*P* < 0,05.

registration of the amperometric trace. Even in these experimental conditions the uncoupling effect of WGA and PHA (comparable to the FCCP effect, Fig. 1 supplementary D) has been confirmed (Fig. 7), while LCA was still ineffective. To attest the uncoupled status between oxygen consumption and ATP synthesis, the membrane potential was measured using the fluorescent probe TMRM, which highlights only the actively respiring membranes. As shown in Fig. 8, in the presence of WGA or PHA the TMRM fluorescent signal is lower than in the untreated sample, indicating that lectins inhibit the formation of the proton gradient, necessary for the ATP synthesis through the  $F_0$ - $F_1$  ATP synthase. LCA does not modify membrane potential (data not shown).

To clarify whether in the alteration of aerobic metabolism induced by WGA or PHA could be involved Nox, syk and PI3K, platelets have been preincubated with 2TFP, LY294002 or piceatannol. In the presence of these inhibitors the oxygen consumption and the ATP synthesis are normalized both in the presence of pyruvate + malate (Fig. 9A and B) or succinate (Fig. 9D and E) with the consequent improvement of the P/O ratio (Fig. 9C,F).

### 3.7. Effect of lectins on platelet viability

Platelets treatment with varying concentrations of WGA and PHA produces a dose- and time-dependent inhibition of L-lactate production (Fig. 10A and B) and a corresponding dose- and time-dependent increase of platelet L-lactic dehydrogenase release (Fig. 10C and D) with a peak at 10  $\mu$ g/mL and 15 min. LCA has no effect.

## 4. Discussion

Lectins are a heterogeneous group of plant-derived carbohydrate-binding proteins. Some lectins are often resistant to cooking and to digestive enzymes and may cause local toxic and inflammatory responses [40]. A variety of diseases can be induced by lectins, such as coeliac disease, type I diabetes, rheumatoid arthritis and respiratory infections [41]. In particular, in coeliac disease the duodenal mucosal damage is due to gluten which contains WGA [42]. In addition, bacterial overgrowth by indigenous microflora in the phytohemagglutinin-fed rat was reported [43]. Various effects of lectins in cells have been described. It was shown that WGA induces monocyte-mediated tumour cell killing [44], IL2 production and IL2 receptor expression in lymphocytes [45], syk activation in porcine splenocytes [46] and Nox activity in neutrophils [47]. Moreover, PHA was shown to induce apoptosis in carcinoma cells [48,49], has anti-cancer properties [50], stimulates NO formation in macrophages [51] and inhibits electrogenic  $Na^+$  absorption in colon and trachea [52]. In human platelets WGA acts as a potent agonist stimulating protein-tyrosine phosphorylation, phosphorylation/activation of PLC $\gamma$ 2 and increase of intracellular  $Ca^{2+}$ , being the pathways src/syk and PI3K/BTK involved in these actions [28,53,54]. PHA induces platelet aggregation too, but with minor potency than WGA, even if the mechanisms involved seem to be the same [28]. In this study, we have shown that WGA and, with minor potency, PHA stimulate ROS and superoxide anion formation, behaving as true platelet agonists [15–18] while LCA is ineffective. As expected, lipid peroxidation is also increased in platelets treated with WGA and

PHA but not with LCA (Fig. 3A and B). It is known that ROS regulate signalling pathways involved in physiological and pathological processes [3,13]. Moreover, lipid peroxidation, strictly connected to cellular oxidative stress, induces various pathogenic intracellular signals leading to cellular dysfunctions through specific aldehyde derivatives such as 4-hydroxynonenal and malondialdehyde. Lipid peroxidation increases  $Ca^{2+}$  levels in cultured endothelial cells [55] and smooth muscle cells [56,57] stimulates PLC [58] and PLD [59] in cultured vascular smooth muscle cells, activates protein kinase C and induces tyrosine phosphorylation [60,61] and activation of epithelial growth factor receptors [62]. In addition, in this study we have shown that WGA and PHA induce a significant decrease in GSH and membrane SH-group content (Fig. 4) indicating a depletion of the platelet antioxidant defence.

One of the main sources of ROS is Nox, ubiquitous in all cells whereby several isoforms have been described. Human platelets express Nox1 and Nox2 [63] and these isoforms play different roles in platelet activation pathways and in thrombosis [64]. Data reported in this study put in evidence that Nox is a relevant source of ROS in platelets treated with WGA and/or PHA as demonstrated by the strong inhibition observed in DTT, DPI and apocynin pre-treated platelets (Figs. 1–2). Nox1 seems to play a pivotal role in ROS formation as 2TFP, that is considered a Nox1 specific inhibitor, decreases superoxide anion and total ROS formation of about 60–70% (Figs. 1–2). Nox1 dependent ROS generation has a main role in cell signalling, cell growth, angiogenesis, motility and blood pressure regulation [65–67]. On the other hand ROS contribute to many diseases, such as cancer, atherosclerosis, hypertension and inflammation [68,69]. ROS generated by Nox facilitate the activation of the syk/PLC $\gamma$  pathway, with consequent  $Ca^{2+}$  mobilization and elevation leading to PKC activation and, in turn, to ROS elevation [28,64,70]. Lectin-induced ROS increase is partially inhibited by piceatannol and LY294002 indicating that the syk/PI3K pathway could be downstream in lectin effect. Therefore, a syk-independent and a syk-dependent ROS production could be suggested [71,72]. However, platelet ROS formation induced by WGA or PHA, besides Nox1 activation, could be due partially to other cellular enzymes such as myeloperoxidase, xanthine oxidase, superoxide dismutase, nitric oxide synthase, cytochrome P450 and mitochondrial oxidases or to the uncoupled aerobic respiration. Concerning the last mechanism, since rotenone or antimycin A, inhibitors of respiratory chain complexes I and III, have no effect, ROS produced by the downstream oxidative phosphorylation are poorly involved in DCFH oxidation induced by lectins. In this study, we have shown that WGA and PHA produce an increase in oxygen consumption, but a decrement in the ATP formation, in a time- and dose-dependent manner. This determines a significant impairment of P/O value (indicative of oxidative phosphorylation coupling), similarly to that obtained in the presence of the uncoupling agent FCCP.

Thus, WGA and to a lesser extent PHA behave as uncoupling agents dissociating the synthesis of ATP from the electron transport chain. To strengthen this data, we have measured the membrane potential in platelets treated with lectins. As reported in Fig. 8 this parameter is decreased, indicating that lectins modify membrane structure and function and greatly decrease energy availability, favouring the uncoupled status and the relative inefficiency of the aerobic machinery. Thus, lectins, which are potent agonists, through the induction of platelet oxidative stress could contribute to the pathogenesis of some cardiovascular dysfunctions and may be involved in inflammatory processes associated with vessel injury.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101456>.

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