ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 4, 2004

Forum Original Research Communication

Functional Role of NADPH Oxidase in Activation of Platelets

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ABSTRACT

Involvement of phagocyte NADPH oxidase in host defense response is well established. In contrast, little is known about the functional role of NADPH oxidase in platelets. In this study, we analyzed involvement of platelet NADPH oxidase in aggregation of human platelets and in amplification of production of reactive oxygen species (ROS) by activated human neutrophils. Apocynin, a known NADPH oxidase inhibitor, as well as superoxide dismutase mimetic Mn(III)tetrakis(1-methyl-1-pyridyl)porphyrin, inhibited ROS generation by collagen-activated platelets, collagen-induced aggregation of platelets, as well as collagen-induced release of thromboxane B_2. These data suggest the key role of intracellular ROS derived from NADPH oxidase in the control of thromboxane A_2 (TXA_2) production in platelets stimulated by collagen. Apocynin also inhibited thrombin-induced ROS production and thrombin-induced platelet aggregation. Activation of neutrophils with latex resulted in an outburst of ROS that was inhibited by apocynin. ROS production by latex-stimulated platelets was modest and also inhibited by apocynin. However, when a mixture of platelets and neutrophils was stimulated with latex, ROS production was three to six times higher in comparison with activation of neutrophils alone. Platelet-dependent augmentation of neutrophil ROS production was abrogated by TXA_2 synthase inhibitor (furegrelate, 1 μ*M*) or by aspirin (300 μ*M*). In summary, NADPH oxidase in platelets seems to play a major role as an intracellular signaling mechanism in the activation of platelets. However, in host defense response involving neutrophils and platelets, platelets enhance ROS production by neutrophils and possibly their cytotoxic potential via the release of TXA_2, which in turn in platelets is not affected by the extracellular release of free radicals. *Antioxid. Redox Signal.* 6, 691–698.

INTRODUCTION

It is well known that phagocytes utilize reactive oxygen species (ROS) produced by the NADH/NADPH oxidase system for the killing of engulfed microorganisms. Recently, it has become apparent that NAD(P)H oxidase is expressed not only in phagocytes, but also in various cell types of the cardiovascular system, such as endothelial cells, smooth muscle cells, fibroblasts, or cardiomyocytes (8).

The vascular NAD(P)H oxidases differ from the phagocytic one in several aspects. The neutrophil NAD(P)H oxidase releases large amounts of superoxide anion (O_2^-) in bursts, whereas the vascular NAD(P)H oxidases continuously produce low levels of O_2^-. Phagocyte-type NAD(P)H oxidase produces O_2^- extracellularly as a part of the host defense response, whereas O_2^- produced by vascular NAD(P)H oxidase seems to serve as an autocrine transduction messenger (8). Indeed, NAD(P)H oxidase-derived ROS modify redox-sensitive signaling pathways and mediate proliferation of smooth muscle cells (14), hypertrophy of cardiomyocytes (3, 41), angiogenesis (39), as well as inflammatory response of endothelium (11).

Phagocytic NAD(P)H oxidase is a multicomponent enzyme, whose activation depends on the migration of cytosolic

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components (p47^{phox}, p67^{phox}, and Rac) (2) to the cellular membrane and coupling to the catalytic cytochrome *b* membrane proteins (gp91^{phox}, p22^{phox}). In vascular cells, such an activation pattern has not been clearly demonstrated and the cytosolic proteins seem to exert a modulatory effect on oxidase activity. Additionally, besides p22^{phox}, vascular cells differentially express gp91^{phox} analogues known as the nonphagocytic NAD(P)H oxidase subunits (8).

Activated platelets are known to generate ROS, such as O_2^-, hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$). Although many ROS sources could be evoked, recent reports demonstrated an NADPH oxidase activity in platelets, while suggesting expression of some of the components of the NAD(P)H oxidase such as p22^{phox}, p67^{phox} (34), p47^{phox}, and small G-protein Rac, but not gp91^{phox} (17, 35). However, a functional role for such activity is still lacking. It was suggested that, similar to neutrophils, platelet-derived ROS are involved in host defense and contribute to killing of bacteria or parasites (9). Antioxidants such as *N*-acetylcysteine inhibit aggregation of platelets (1); therefore, it is possible that platelet-derived O_2^- modifies platelet function by direct effects on redox-sensitive sites (7, 26) or by modifying nitric oxide availability (31). Furthermore, some evidence was provided that NADPH oxidase-dependent platelet O_2^- release was not involved in initial platelet aggregation, but rather in secondary platelet recruitment, most likely by mechanisms involving inhibition of platelet ectonucleotidase (17).

Thus, there is little evidence on the functional role of O_2^- produced by NAD(P)H oxidase in platelets. The present study was undertaken to examine the role of platelet NADPH oxidase in two experimental models relevant to hemostasis and to host defense response. We studied the effects of NAD(P)H inhibition on platelet aggregation induced by collagen, ADP, and thrombin and examined a contribution of platelet-derived free radicals to the amplification of respiratory burst activity of neutrophils.

MATERIALS AND METHODS

Platelet and neutrophil isolation

Venous blood was obtained from human volunteers in University Hospital Blood Bank Center. Volunteer donors had not taken any medicines for the preceding 2 weeks. Blood was anticoagulated with sodium citrate (3.2%; 1:9, vol/vol).

To obtain platelet-rich plasma (PRP), blood was centrifuged at 250 *g* for 20 min. Platelet-poor plasma (PPP) was obtained by centrifugation of remaining blood for 5 min at 2,000 *g*. To obtain washed platelets (WP), platelets were washed twice in prostaglandin I_2 containing phosphate-buffered saline (PBS) according to Radomski and Moncada (30) and finally suspended (2×10^8 platelet/ml) in Ca^{2+}-free PBS containing 0.1% albumin. Filtered platelets (FP) were obtained by gel filtration on Sepharose 2B columns. Contamination of neutrophils in PRP and WP was less than $1/10^6$ and $1/10^8$, respectively, and in FP it was not detectable.

Polymorphonuclear neutrophils (PMNs) were isolated as follows: a sample of erythrocytes and leukocytes was mixed with 3% solution of dextran (1:1, vol/vol) and allowed to sediment for 30 min at 37°C. After sedimentation of erythrocytes, the upper fraction, which contained PMNs and monocytes, was used for Ficoll-gradient isolation of PMNs according to Boyum (5). Residual erythrocytes were removed by means of hypoosmotic lysis with ice-cold 0.2% NaCl solution. Finally, 2×10^6 leukocytes were suspended in Ca^{2+}-free PBS containing 0.1% albumin. More than 95% of the isolated leukocytes were neutrophils in microscope evaluation. Cells were viable in >98% as evidenced by trypan blue staining.

Platelet aggregation

Platelet aggregation was studied in PRP in a model of dual-channel Chrono-log aggregometer using Born's method (4). Baseline on the aggregometer was set using PRP, whereas PPP was used to set full transmittance. PRP (500 μl) was equilibrated at 37°C for 3 min with continued stirring at 1,100 rpm and then stimulated with collagen, ADP, or thrombin. In the beginning of each experiment, submaximal concentrations for collagen, ADP, and thrombin were determined. They were in the range of 0.3–1.2 μg/ml, 3–7 μ*M*, and 0.03–0.3 U for collagen, ADP, and thrombin, respectively.

Functional effects of apocynin, superoxide dismutase (SOD), or the cell-permeable SOD mimetic Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin [Mn(III)TMPyP] on platelet aggregation were studied using submaximal concentration of aggregating compounds. Inhibitors were added 2 min before stimulation of platelets with the aggregating compound. Inhibition of collagen-induced aggregation by apocynin was similar in PRP, WP, and FP, indicating a negligible contribution of NADPH oxidase from neutrophils to platelet aggregation in all three types of platelet preparations. Thus, only data from experiments in PRP are reported.

Measurements of thromboxane B_2 (TXB_2)

TXB_2 measurements were performed in the samples taken from the aggregation experiments. Following measurement of peak aggregation (8 min after addition of collagen), samples were treated with indomethacin (5 μ*M*) to prevent any further TXB_2 formation and placed on ice (2–4°C). Samples were then transfered to Eppendorff tubes, centrifuged at 300 *g* for 3 min at room temperature, and stored at −20°C until assayed. TXB_2 levels were determined in duplicates in aliquots from samples using a commercially available ELISA kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.). TXB_2 levels were expressed in nanograms per milliliter.

Free radical production by platelets alone and by latex-stimulated neutrophils in the absence and in the presence of platelets

To assess levels of ROS production by collagen- or thrombin-stimulated platelets, lucigenin-enhanced chemiluminescence was used. The assay was performed in a plate luminometer (FARCyte Fluorescence-Luminescence Plate Reader; Tecam/Amersham Biosciences Corp., Piscataway, NJ, U.S.A.). Each well of the 96-well plate contained 1×10^8 platelets/ml, resuspended in Krebs buffer, pH 7.4, and 5 μ*M* lucigenin.When indicated, the agonists collagen (1.2 μg/ml) and thrombin (0.3 U) were added in the absence or presence

of apocynin (600 μ*M*), diphenyleneiodonium (DPI; 20 μ*M*), SOD (100 U/ml), or SOD mimetic [Mn(III)TMPyP, 20 μ*M*]. Final volume was adjusted to 250 μl. Inhibitors were added 2 min before platelet agonists (collagen or thrombin).

To assess levels of ROS production by latex-stimulated neutrophils in the absence and in the presence of platelets, luminol-enhanced chemiluminescence was used (1 m*M*). The assay was carried out in the automatic microplate luminometer Autolumat Berthold EG&G model LB 96A (Wildbad, Germany) as described previously (20). ROS production by neutrophils alone or by neutrophils and platelets was measured in the absence and in the presence of the following inhibitors: apocynin (10–600 μ*M*), DPI (1–100 μ*M*), aspirin (300 μ*M*), or furegrelate (1 μ*M*). Isolated neutrophils, platelets, and reagents were added into individual Nunc (Roskilde, Denmark) nontransparent microplate wells in the following order: 150 μl of luminol (Serva; 5-amino-2,3-dihydro-1,4-phthalazinedione) in Krebs buffer; 50 μl of neutrophils suspended in PBS at a concentration of 10^6/ml; 25 μl of platelets suspended in PBS, at a concentration of 2×10^8/ml; inhibitor in a volume of 10 μl; and finally 1% dry content latex (0.9 μm in diameter, 2.5×10^{10} particles/ml). In the absence of platelets or an inhibitor, saline was added so that the total reaction volume was always 245 μl in each well. All measurements were run in duplicates. Basal chemiluminescence of nonstimulated neutrophils and/or platelets was also included in each assay.

In all assays, chemiluminescence was measured for 40 min at 37°C, and readings were expressed as relative luminescence units (RLU) or as an integrated RLU. The former were calculated from the height of the peak of the luminescence, and the latter was measured as an area under the curve of RLU recorded over the 40-min period. Both parameters were highly correlated in all performed experiments. The integral of the signal was chosen for analysis. As there was a considerable variability in magnitude of ROS generation between samples from various donors, data are presented as percentage of the count of nonstimulated platelets or as percentage of latex-stimulated neutrophil response in a given experiment.

Immunoblotting of gp91^{phox} in neutrophils and platelets

Suspensions of filtered platelets and isolated neutrophils from the same healthy volunteers were centrifuged at 10,000 *g* for 5 min at 4°C, followed by pellet lysis in lysis buffer [1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) in PBS containing 1 m*M* phenylmethylsulfonyl fluoride, 100 μ*M* leupeptin, 50 μ*M* pepstatin A]. Protein concentration of lysates was determined using the Bradford method. Samples containing equal amounts of total protein were mixed with gel loading buffer (50 m*M* Tris, 3% SDS, 10% glycerol, 7% 2-mercaptoethanol, 0.1% bromophenol blue) in a ratio of 4:1 (vol/vol) and boiled (4 min). Then samples (50 μg of total protein per lane) were separated on 7.5% SDS–polyacrylamide gels (Mini Protean II, Bio-Rad, Hercules, CA, U.S.A.) using the Laemmli buffer system, and proteins were semidry transferred to nitrocellulose membranes (Bio-Rad). Nonspecific binding sites were blocked overnight at 4°C with 5% nonfat dried milk, and membranes were incubated 5 h at room temperature with rabbit polyclonal antibody to gp91^{phox} (1:500) (Upstate, Biotechnology, Lake Placid, NY, U.S.A.). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (2 h at room temperature, 1:5,000; Upstate), and signal intensities were measured using an enhanced chemiluminescence detection system (ECL, Amersham, Arlington Heights, IL, U.S.A.). Additionally, membranes were reprobed with monoclonal anti-β-actin antibody (1:5,000; Sigma, St. Louis, MO, U.S.A.). Prestained markers (Amersham) were used for molecular weight determinations. Protein bands were scanned and analyzed with freeware Scion image (Scion Cor., Frederick, MD, U.S.A.). The data were normalized to constitutively expressed β-actin protein.

Reagents and drugs

Collagen was obtained from Chrono-log (U.S.A.), thrombin from Polfa-Krakow (Krakow, Poland), aspirin from Bayer (Leverkusen, Germany), DPI, SOD, and apocynin (4 hydroxy-3′-methoxyacetophenone) from Sigma–Aldrich Chemicals International, and furegrelate sodium from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, U.S.A.). Latex was obtained from Instytut, Katalizy, PAN (Krakow, Poland). SOD mimetic Mn(III)TMPyP was from Cayman.

Data analysis

Results are expressed as means ± SD. Differences between means were evaluated by the unpaired Student's *t* test. A *p* value of <0.05 was considered statistically significant.

RESULTS

Role of NADPH oxidase in platelet aggregation

Apocynin, the NADPH oxidase inhibitor, inhibited platelet aggregation induced by collagen, ADP, or thrombin in a concentration-dependent manner (Fig. 1). The inhibitory effect of apocynin was most pronounced in collagen-induced

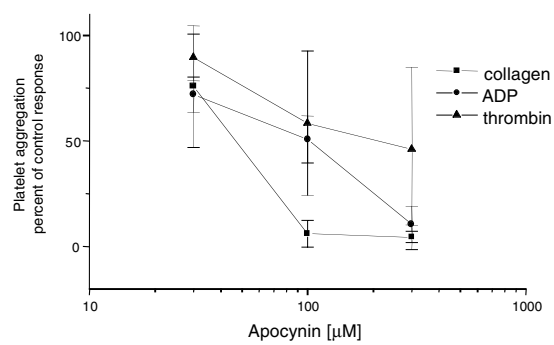

FIG. 1. Concentration-dependent inhibition of platelet aggregation by the NADPH oxidase inhibitor, apocynin in human PRP. Platelet aggregation was induced by submaximal concentration of collagen (0.3–1.2 μg/ml), ADP (3–7 μ*M*), or thrombin (0.03–0.3 U). Data are presented as means ± SD from at least five experiments.

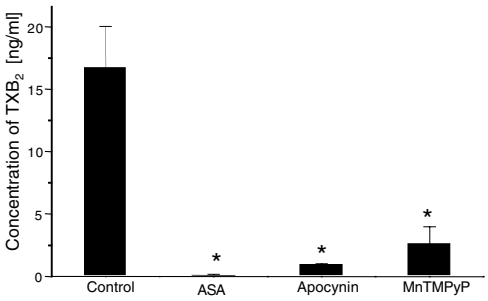

FIG. 2. Inhibition of collagen-induced TXB_2 production by apocynin (300 μ*M*), by cell-permeable SOD mimetic [Mn(III)TMPyP; 100 μ*M*], and by aspirin (ASA; 300 μ*M*) in human PRP. Data are presented as means ± SD from three to five experiments. *$p < 0.05$ versus control response.

aggregation and was associated with the suppression of collagen-induced formation of TXB_2 (Fig. 2). Also, the SOD mimetic Mn(III)TMPyP (100 μ*M*) inhibited collagen-induced aggregation of platelets (by >90%) and TXB_2 formation (Fig. 2). On the other hand, SOD (100 U/ml) did not modify either collagen-induced platelet aggregation or collagen-induced TXB_2 release (data not shown).

Activation of platelets with collagen (1.2 μg/ml) or thrombin (0.3 U) resulted in augmentation of ROS production as measured by lucigenin-enhanced chemiluminescence (Fig. 3). ROS production by platelets in basal conditions, as well as after stimulation with collagen or thrombin, was inhibited by apocynin (600 μ*M*) and MnTMPyP (20 μ*M*), but not by SOD (100 U/ml) (Fig. 3). Also DPI (20 μ*M*) diminished by 63% basal ROS production by platelets and abrogated an increase in ROS production in collagen- or thrombin-stimulated platelets.

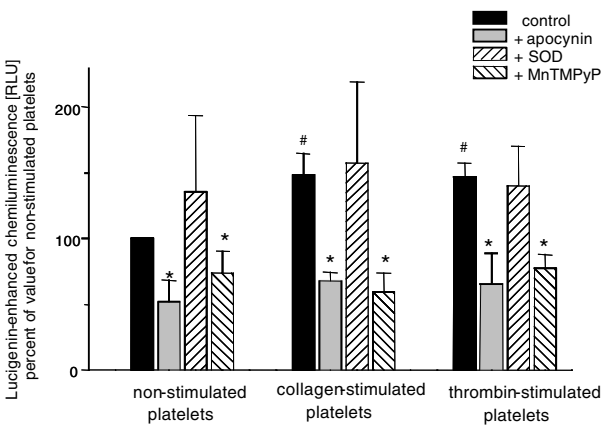

FIG. 3. Inhibition of platelet-derived ROS production by apocynin (600 μ*M*) or by cell-permeable SOD mimetic [Mn(III)TMPyP, 20 μ*M*], but not by SOD (100 U/ml) as measured by lucigenin-enhanced chemiluminescence. Data are presented as means ± SD from four to six experiments. *$p < 0.05$ versus respective control value; #$p < 0.05$ versus nonstimulated platelets.

Expression of gp91^{phox} in platelets and neutrophils

A major subunit of NADPH oxidase, gp91^{phox} protein, was detected in platelets and in neutrophils (~70 kDa). Interestingly, the level of expression of gp91^{phox} in platelets and neutrophils was similar, if calculated in relation to expression of β-actin (Fig. 4).

Role of thromboxane A_2 (TXA_2) in platelet-dependent amplification of ROS production by neutrophils

Latex-stimulated activation of neutrophils (10^6/ml) resulted in an outburst of ROS [from 67,800 ± 3,433 to 3,053,000 ± 656,810 (integrated RLU)] as measured by luminol-enhanced chemiluminescence (Fig. 5A). Latex-stimulated response of neutrophils was inhibited by >90% by apocynin (100–600 μ*M*) or by DPI (10–100 μ*M*) (data not shown). In contrast with neutrophils, stimulation of platelets (2×10^8/ml) with latex resulted in modest augmentation of ROS production as detected by luminol-enhanced chemiluminescence [from 11,673 ± 4,331 to 37,703 ± 27,739 ($n = 6$) (integrated RLU)]. This response was also completely inhibited by 300 μ*M* apocynin [10,453 ± 3,030 ($n = 6$) (integrated

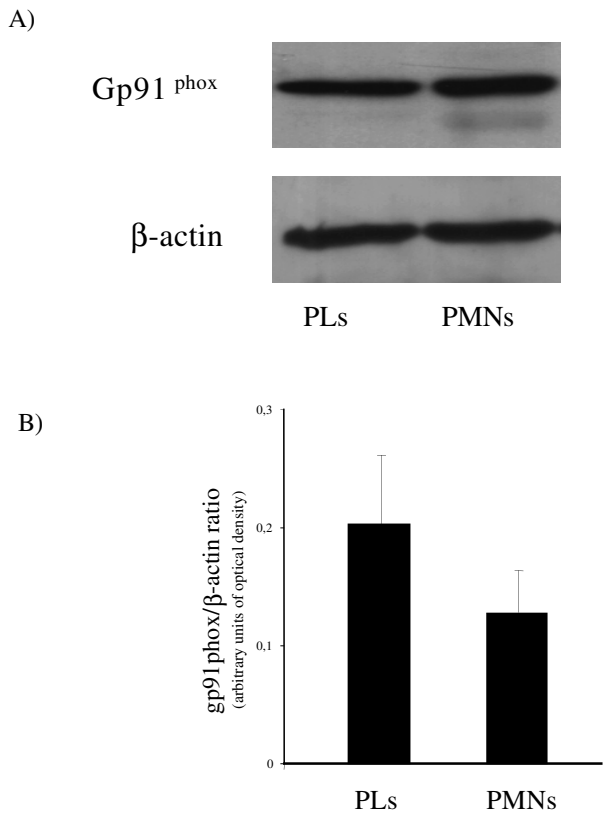

FIG. 4. (A) Representative immunoblotting of gp91^{phox} protein and β-actin protein in lysates of gel-filtered platelets (PLs) and isolated neutrophils (PMNs). **(B)** Densitometric analysis of gp91^{phox} protein expression in platelets and neutrophils isolated simultaneously from blood of healthy volunteers ($n = 3$). The data are normalized to β-actin protein.

RLU)]. Importantly, in the presence of platelets (2×10^8/ml), latex-induced production of free radicals by neutrophils (10^6/ml) was three to six times higher as compared with that produced by activated neutrophils alone (Figs. 5 and 6). Inhibition of TXA_2 synthase (furegrelate, 1 μ*M*) did not change the response of neutrophils, whereas the inhibitor of cyclooxygenase (COX), (aspirin, 300 μ*M*) potentiated the response of neutrophils (Figs. 5 and 6). However, if neutrophils were stimulated in the presence of platelets together with furegrelate or aspirin, each of them abrogated platelet-dependent augmentation of the neutrophil response (Figs. 5 and 6).

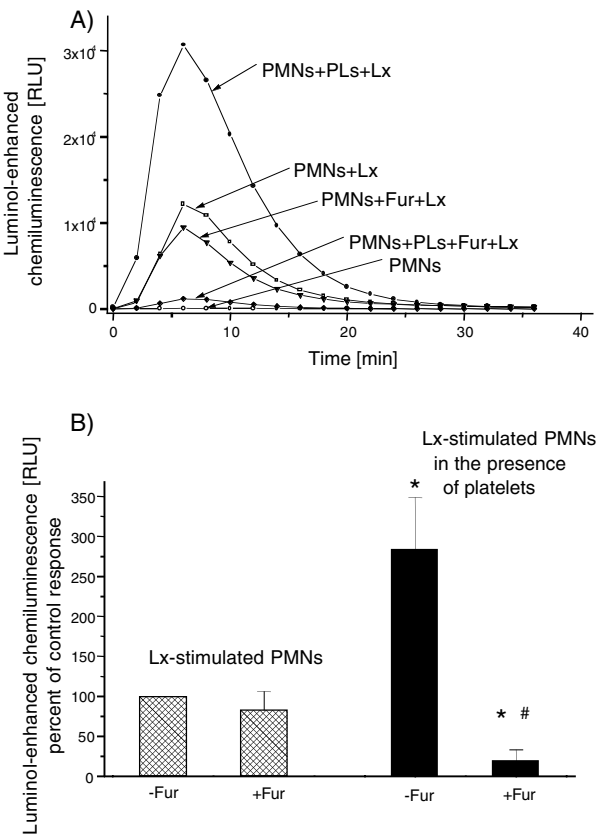

FIG. 5. Amplification of latex (Lx) induced ROS production in neutrophils (PMNs) in the presence of platelets (PLs) and its inhibition by TXA_2 synthase inhibitor (Fur, furegrelate; 1 μ*M*). (A) Time course of the responses in the representative experiment. **(B)** Magnitude of the response measured as the integral of luminol-enhanced chemiluminescence. Data are presented as means ± SD from four experiments. $*p <$ 0.05 versus latex-stimulated PMNs; $^{\#}p < 0.05$ versus latex-stimulated PMNs in the presence of platelets.

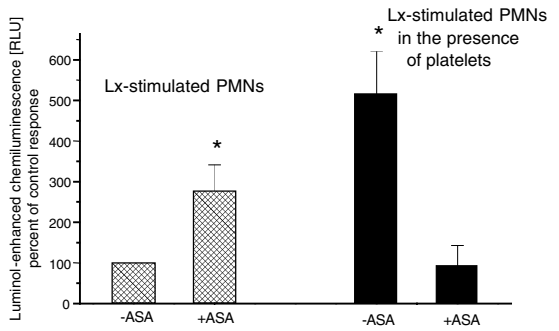

FIG. 6. Effects of aspirin (ASA) on the amplification of latex (Lx)-induced ROS production by neutrophils (PMNs) in the presence of platelets. Data are presented as means ± SD from six experiments. $*p < 0.05$ versus latex-stimulated PMNs; $^{\#}p < 0.05$ versus latex-stimulated PMNs in the presence of platelets.

DISCUSSION

In the present study, we demonstrate that apocynin [an inhibitor of NAD(P)H oxidase] and a cell-permeable SOD mimetic profoundly inhibited aggregation of human platelets induced by collagen and collagen-induced release of TXB_2. Platelet aggregation induced by ADP or by thrombin was less affected by apocynin. On the other hand, we demonstrate that $gp91^{phox}$ protein was expressed in platelets at a level comparable to that of neutrophils. However, activated platelets, in contrast to activated neutrophils, produced relatively low levels of NADPH oxidase–derived ROS that could be detected by chemiluminescence. Interestingly, in the presence of platelets, respiratory burst of neutrophils and possibly their cytotoxic potential were strongly potentiated by a mechanism involving platelet-derived TXA_2, which in turn was sensitive in platelets to apocynin and to the cell-permeable SOD mimetic, but not to SOD itself. These data suggest that the ROS generated by NADPH oxidase in platelets represent an important intracellular signaling mechanism for platelet activation/aggregation rather than a direct mechanism for killing of microorganisms (9).

Over 20 years ago, it was shown that platelets released O_2^- (19). Possible enzymatic sources of ROS in platelets include COX (7), mitochondrial enzymes (33), xanthine oxidase (24), and NAD(P)H oxidase (17). The last one seems to be the major source of ROS in platelets. Indeed, O_2^- production by human platelets stimulated by phorbol ester (*O*-tetradecanoylphorbol 13-acetate) or calcium ionophore was entirely dependent on NADPH oxidase (34). Numerous studies analyzed the effects of exogenous ROS on the function of platelets (28, 31) or chemical identity of ROS produced by platelets (7, 15, 16, 26). It was suggested that platelet-derived ROS were involved in host defense response and killing of bacteria or parasites (9). On the other hand, H_2O_2 was suggested to mediate collagen-induced aggregation, although the source of H_2O_2 production was not identified (26). The role of NADPH oxidase–like enzyme in platelet aggregation was previously suggested by Salvemini *et al.* (32) on the basis of the inhibitory effect of DPI, a broad-spectrum flavoprotein inhibitor (37) on ADP- or thrombin-induced platelet aggregation. Later, however, it was demonstrated that O_2^- derived from NADPH oxidase was involved in the recruitment of additional platelets to thrombus formation, but not in primary platelet aggregation (17).

Here we show that apocynin [a selective inhibitor of NAD(P)H oxidase] abrogated platelet aggregation induced by

collagen and significantly attenuated platelet aggregation induced by ADP or thrombin. Apocynin, as well as DPI, inhibited ROS production by activated platelets, suggesting that the antiplatelet effect of apocynin was due to NAPDH oxidase inhibition. We also demonstrate that SOD, which has restricted penetration into cells and wipes off only extracellular O_2^-, did not modify ROS production by platelets, collagen-induced aggregation, and collagen-induced TXA_2 release. On the other hand, Mn(III)TMPyP, (a cell-permeable SOD mimetic) blunted ROS production by platelets, collagen-induced platelet aggregation, and collagen-induced TXA_2 release. These results suggest the following: first, the involvement of an intracellular, but not extracellular, target for NAD(P)H oxidase dependent regulation of platelet function and, second, obligatory involvement of O_2^- in this regulation. A possible involvement of other types of ROS in this regulation remains to be defined.

Our findings that collagen-induced aggregation was most sensitive to the inhibitory effect of apocynin, followed by ADP- and thrombin–induced aggregation, seem to correlate with the known contribution of TXA_2 to collagen-, ADP-, and thrombin-induced aggregation of platelets.

Noteworthy, it was demonstrated that oxygen species could activate arachidonic release *via* direct stimulation of phospholipase A_2 (42). Furthermore, peroxide tone was suggested to be necessary for the activation of COX (38). NADPH oxidase could be involved in activation of the pathway leading to generation of TXA_2 in platelets at the level of phospholipase A_2 or COX-1, or possibly through other targets, *e.g.,* tyrosine kinase (18) or protein kinase C (22, 29). Obviously, upstream and downstream mediators of NADPH oxidase in platelets remain to be defined. Nevertheless, our data support the notion that NADPH oxidase-derived ROS are critically involved in platelet activation. This seems to be in line with the known ability of antioxidants, such as *N*-acetylcysteine, to inhibit platelet aggregation (1).

A large body of evidence indicates that the role of platelets is not limited to the regulation of hemostasis and thrombosis. In particular, direct bridging of platelets with leukocytes, dependent on specific interactions between adhesion molecules on platelet and leukocyte, play an important role in host defense response (21). The mechanisms of platelet–neutrophil interaction involves P-selectin and glycoprotein IIb/IIIa in platelets and P-selectin glycoprotein ligand (PSGL-1) and CD11b/CD18 in neutrophils (6, 12, 13, 36). Importantly, platelet–leukocyte complexes represent a large subpopulation of neutrophils with a greater capacity of phagocytosis and ROS production (25). Indeed, our data showed that in the presence of platelets, respiratory burst of neutrophils was potentiated, as shown previously (43). Here, we also demonstrate that augmented ROS production by neutrophils in the presence of platelets was abrogated by the inhibitor of TXA_2 synthase (furegrelate) or by the inhibitor of COX-1 (aspirin). Accordingly, this phenomenon involves COX-1-derived TXA_2 from platelets.

We did not provide direct evidence whether furegrelate acted on neutrophils or platelets in our experimental setting. However, human neutrophils, in contrast to platelets, do not possess TXA_2 synthase (23); therefore, it seems unlikely that a direct effect of TXA_2 synthase inhibitor on neutrophils is involved. Interestingly, aspirin augmented the latex-induced neutrophil response, suggesting that COX-1 is not coupled to TXA_2 synthase in neutrophils, but rather to prostaglandin E (PGE) synthase. Indeed, neutrophils produced a substantial amount of PGE_2, and PGE_2 is known to inhibit neutrophils (27, 40). Accordingly, inhibition of COX-1 in neutrophils by aspirin most likely results in diminished PGE_2 production and augmentation of neutrophil response.

Taken together, our results suggest the role of TXA_2 in platelet-dependent augmentation of respiratory burst of neutrophils. This seems to correlate with our previous findings on the key role of lipid mediators in platelet–neutrophil interactions (10).

In summary, we point out that NADPH oxidase in platelets plays a major role as an intracellular signaling mechanism in platelet activation. Additionally, in host defense response involving neutrophils and platelets, platelets enhance production of free radicals by neutrophils and possibly their cytotoxic potential through the release of TXA_2, and not by the extracellular release of ROS.

Perspectives

Presently, it is considered that NAD(P)H oxidase is a major source of ROS in the vascular wall. Inhibition of ROS overproduction may prove effective in the prevention or treatment of a variety of cardiovascular diseases, such as hypertension, atherosclerosis, or heart failure. As we show here, inhibition of NADPH oxidase in platelets offers also antiplatelet effects.

ACKNOWLEDGMENTS

We would like to thank Lena Stelmach, Jolanta Reyman, Renata Budzynska, Lukasz Mateuszuk, Joanna Dracz, and Katarzyna Zajac for their excellent technical assistance. This work was supported by KBN grant no. 3PO5A 003 25.

ABBREVIATIONS

ADP, adenosine diphosphate; COX, cyclooxygenase; DPI, diphenyleneiodonium; FP, filtered platelets; H_2O_2, hydrogen peroxide; Mn(III)TMPyP, Mn(III) tetrakis(1-methyl-4-pyridyl) porphyrin; O_2^-, superoxide anion; PBS, phosphate-buffered saline; PGE, prostaglandin E; PMNs, polymorphonuclear neutrophils; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RLU, relative luminescence units; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; WP, washed platelets.

REFERENCES

1. Anfossi G, Russo I, Massucco P, Mattiello L, Cavalot F, and Trovati M. *N*-Acetyl-L-cysteine exerts direct anti-aggregating effect on human platelets. *Eur J Clin Invest* 31: 452–461, 2001.

2. Babior BM. NADPH oxidase: an update. *Blood* 93: 1464–1476, 1999.
3. Bendall JK, Cave AC, Heymes C, Gall N, and Shah AM. Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. *Circulation* 105: 293–296, 2002.
4. Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nauchni Tr Vissh Med Inst Sofiia* 194: 927–929, 1962.
5. Boyum A. Isolation of leucocytes from human blood. Further observations. Methylcellulose, dextran, and ficoll as erythrocyteaggregating agents. *Scand J Clin Lab Invest Suppl* 97: 31–50, 1968.
6. Brown KK, Henson PM, Maclouf J, Moyle M, Ely JA, and Worthen GS. Neutrophil-platelet adhesion: relative roles of platelet P-selectin and neutrophil beta2 (DC18) integrins. *Am J Respir Cell Mol Biol* 18: 100–110, 1998.
7. Caccese D, Pratico D, Ghiselli A, Natoli S, Pignatelli P, Sanguigni V, Iuliano L, and Violi F. Superoxide anion and hydroxyl radical release by collagen-induced platelet aggregation—role of arachidonic acid metabolism. *Thromb Haemost* 83: 485–490, 2000.
8. Cai H, Griendling KK, and Harrison DG. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci* 24: 471–478, 2003.
9. Cesbron JY, Capron A, Vargaftig BB, Lagarde M, Pincemail J, Braquet P, Taelman H, and Joseph M. Platelets mediate the action of diethylcarbamazine on microfilariae. *Nature* 325: 533–536, 1987.
10. Chlopicki S, Lomnicka M, and Gryglewski RJ. Obligatory role of lipid mediators in platelet-neutrophil adhesion. *Thromb Res* 110: 287–292, 2003.
11. De Keulenaer GW, Ushio-Fukai M, Yin Q, Chung AB, Lyons PR, Ishizaka N, Rengarajan K, Taylor WR, Alexander RW, and Griendling KK. Convergence of redox-sensitive and mitogen-activated protein kinase signaling pathways in tumor necrosis factor-alpha-mediated monocyte chemoattractant protein-1 induction in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 20: 385–391, 2000.
12. Diacovo TG, Roth SJ, Buccola JM, Bainton DF, and Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood* 88: 146–157, 1996.
13. Evangelista V, Manarini S, Rotondo S, Martelli N, Polischuk R, McGregor JL, de Gaetano G, and Cerletti C. Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the beta 2 integrin CD11b/CD18. *Blood* 88: 4183–4194, 1996.
14. Gorlach A, Kietzmann T, and Hess J. Redox signaling through NADPH oxidases: involvement in vascular proliferation and coagulation. *Ann N Y Acad Sci* 973: 505–507, 2002.
15. Iuliano L, Pedersen JZ, Pratico D, Rotilio G, and Violi F. Role of hydroxyl radicals in the activation of human platelets. *Eur J Biochem* 221: 695–704, 1994.
16. Iuliano L, Colavita AR, Leo R, Pratico D, and Violi F. Oxygen free radicals and platelet activation. *Free Radic Biol Med* 22: 999–1006, 1997.
17. Krotz F, Sohn HY, Gloe T, Zahler S, Riexinger T, Schiele TM, Becker BF, Theisen K, Klauss V, and Pohl U. NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. *Blood* 100: 917–924, 2002.
18. Lowe GM, Hulley CE, Rhodes ES, Young AJ, and Bilton RF. Free radical stimulation of tyrosine kinase and phosphatase activity in human peripheral blood mononuclear cells. *Biochem Biophys Res Commun* 245: 17–22, 1998.
19. Marcus AJ, Silk ST, Safier LB, and Ullman HL. Superoxide production and reducing activity in human platelets. *J Clin Invest* 59: 149–158, 1977.
20. Miedzobrodzki J and Kaszycki P. Effect of *Staphylococcus aureus* serine proteinase on the respiratory burst in phagocytic cells in vitro. *Acta Microbiol Pol* 49: 237–242, 2000.
21. Nash GB. Adhesion between neutrophils and platelets: a modulator of thrombotic and inflammatory events? *Thromb Res* 74 Suppl 1: S3–S11, 1994.
22. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, and Stowe DF. Reactive oxygen species precede the epsilon isoform of protein kinase C in the anesthetic preconditioning signaling cascade. *Anesthesiology* 99: 421–428, 2003.
23. Nusing R and Ullrich V. Immunoquantitation of thromboxane synthase in human tissues. *Eicosanoids* 3: 175–180, 1990.
24. Pandey NR, Kaur G, Chandra M, Sanwal GG, and Misra MK. Enzymatic oxidant and antioxidants of human blood platelets in unstable angina and myocardial infarction. *Int J Cardiol* 76: 33–38, 2000.
25. Peters MJ, Dixon G, Kotowicz KT, Hatch DJ, Heyderman RS, and Klein NJ. Circulating platelet–neutrophil complexes represent a subpopulation of activated neutrophils primed for adhesion, phagocytosis and intracellular killing. *Br J Haematol* 106: 391–399, 1999.
26. Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP, and Violi F. Hydrogen peroxide is involved in collagen-induced platelet activation. *Blood* 91: 484–490, 1998.
27. Pouliot M, Gilbert C, Borgeat P, Poubelle PE, Bourgoin S, Creminon C, Maclouf J, McColl SR, and Naccache PH. Expression and activity of prostaglandin endoperoxide synthase-2 in agonist-activated human neutrophils. *FASEB J* 12: 1109–1123, 1998.
28. Pratico D, Pasin M, Barry OP, Ghiselli A, Sabatino G, Iuliano L, FitzGerald GA, and Violi F. Iron-dependent human platelet activation and hydroxyl radical formation: involvement of protein kinase C. *Circulation* 99: 3118–3124, 1999.
29. Pricci F, Leto G, Amadio L, Iacobini C, Cordone S, Catalano S, Zicari A, Sorcini M, Di Mario U, and Pugliese G. Oxidative stress in diabetes-induced endothelial dysfunction involvement of nitric oxide and protein kinase C. *Free Radic Biol Med* 35: 683–694, 2003.
30. Radomski M and Moncada S. An improved method for washing of human platelets with prostacyclin. *Thromb Res* 30: 383–389, 1983.
31. Salvemini D and Botting R. Modulation of platelet function by free radicals and free-radical scavengers. *Trends Pharmacol Sci* 14: 36–42, 1993.
32. Salvemini D, Radziszewski W, Mollace V, Moore A, Willoughby D, and Vane J. Diphenylene iodonium, an

inhibitor of free radical formation, inhibits platelet aggregation. *Eur J Pharmacol* 199: 15–18, 1991.
33. Sanner BM, Meder U, Zidek W, and Tepel M. Effects of glucocorticoids on generation of reactive oxygen species in platelets. *Steroids* 67: 715–719, 2002.
34. Seno T, Inoue N, Gao D, Okuda M, Sumi Y, Matsui K, Yamada S, Hirata KI, Kawashima S, Tawa R, Imajoh-Ohmi S, Sakurai H, and Yokoyama M. Involvement of NADH/NADPH oxidase in human platelet ROS production. *Thromb Res* 103: 399–409, 2001.
35. Soulet C, Gendreau S, Missy K, Benard V, Plantavid M, and Payrastre B. Characterisation of Rac activation in thrombin- and collagen-stimulated human blood platelets. *FEBS Lett* 507: 253–258, 2001.
36. Spangenberg P, Redlich H, Bergmann I, Losche W, Gotzrath M, and Kehrel B. The platelet glycoprotein IIb/IIIa complex is involved in the adhesion of activated platelets to leukocytes. *Thromb Haemost* 70: 514–521, 1993.
37. Stuehr DJ, Fasehun OA, Kwon NS, Gross SS, Gonzalez JA, Levi R, and Nathan CF. Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. *FASEB J* 5: 98–103, 1991.
38. Taylor L, Menconi MJ, and Polgar P. The participation of hydroperoxides and oxygen radicals in the control of prostaglandin synthesis. *J Biol Chem* 258: 6855–6857, 1983.
39. Ushio-Fukai M, Tang Y, Fukai T, Dikalov SI, Ma Y, Fujimoto M, Quinn MT, Pagano PJ, Johnson C, and Alexander RW. Novel role of gp91(phox)-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. *Circ Res* 91: 1160–1167, 2002.
40. Wise H. Activation of the prostaglandin EP4-receptor subtype is highly coupled to inhibition of *N*-formyl-methionyl-leucyl-phenylalanine-stimulated rat neutrophil aggregation. *Prostaglandins Leukot Essent Fatty Acids* 58: 77–84, 1998.
41. Xiao L, Pimentel DR, Wang J, Singh K, Colucci WS, and Sawyer DB. Role of reactive oxygen species and NAD(P)H oxidase in alpha(1)-adrenoceptor signaling in adult rat cardiac myocytes. *Am J Physiol Cell Physiol* 282: C926–C934, 2002.
42. Xu J, Yu S, Sun AY, and Sun GY. Oxidant-mediated AA release from astrocytes involves cPLA(2) and iPLA(2). *Free Radic Biol Med* 34: 1531–1543, 2003.
43. Zalavary S, Grenegard M, Stendahl O, and Bengtsson T. Platelets enhance Fc(gamma) receptor-mediated phagocytosis and respiratory burst in neutrophils: the role of purinergic modulation and actin polymerization. *J Leukoc Biol* 60: 58–68, 1996.

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Received for publication January 31, 2004; accepted April 19, 2004.

This article has been cited by:

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2. Ping Song, Ming-Hui Zou. 2012. Regulation of NAD(P)H oxidases by AMPK in cardiovascular systems. *Free Radical Biology and Medicine* **52**:9, 1607-1619. [CrossRef]
3. Stefan Chlopicki, Magdalena Lomnicka, Andrzej Fedorowicz, El#bieta Grochal, Karol Kramkowski, Andrzej Mogielnicki, W#odzimierz Buczko, Roberto Motterlini. 2012. Inhibition of platelet aggregation by carbon monoxide-releasing molecules (CO-RMs): comparison with NO donors. *Naunyn-Schmiedeberg's Archives of Pharmacology* . [CrossRef]
4. Jawahar L. Mehta, Magomed Khaidakov, Paul L. Hermonat, Sona Mitra, Xianwei Wang, Guiseppe Novelli, Tatsuya Sawamura. 2011. LOX-1: A New Target for Therapy for Cardiovascular Diseases. *Cardiovascular Drugs and Therapy* . [CrossRef]
5. Vidhi P. Shah, Hesum A. Chegini, Susan R. Vishneski, Ross V. Weatherman, Peter F. Blackmore, Yuliya Dobrydneva. 2011. Tamoxifen promotes superoxide production in platelets by activation of PI3-Kinase and NADPH oxidase pathways. *Thrombosis Research* . [CrossRef]
6. Andrea L. Sheehan, Samuel Carrell, Bryon Johnson, Bojana Stanic, Botond Banfi, Francis J. Miller. 2011. Role for Nox1 NADPH oxidase in atherosclerosis. *Atherosclerosis* **216**:2, 321-326. [CrossRef]
7. Janahan Dharmarajah, Jane F. Arthur, Christopher G. Sobey, Grant R. Drummond. 2010. The anti-platelet effects of apocynin in mice are not mediated by inhibition of NADPH oxidase activity. *Naunyn-Schmiedeberg's Archives of Pharmacology* **382**:4, 377-384. [CrossRef]
8. Giuliana Leoncini, Maria Grazia Signorello, Alessia Segantin, Enrica Giacobbe, Ugo Armani, Antonietta Piana, Paola Camicione. 2009. In retinal vein occlusion platelet response to thrombin is increased. *Thrombosis Research* **124**:6, e48-e55. [CrossRef]
9. Mohammed El Haouari, Juan A. Rosado. 2008. Platelet signalling abnormalities in patients with type 2 diabetes mellitus: A review. *Blood Cells, Molecules, and Diseases* **41**:1, 119-123. [CrossRef]
10. Katsuhiko Takenaka, Sho-ichi Yamagishi, Takanori Matsui, Kazuo Nakamura, Yuko Jinnouchi, Yumiko Yoshida, Shin-ichiro Ueda, Yoshio Katsuki, Yousuke Katsuda, Tsutomu Imaizumi. 2008. Pigment epithelium-derived factor (PEDF) administration inhibits occlusive thrombus formation in rats: A possible participation of reduced intraplatelet PEDF in thrombosis of acute coronary syndromes. *Atherosclerosis* **197**:1, 25-33. [CrossRef]
11. Jacek Mi#dzobrodzki, Tomasz Panz, Przemys#aw M. P#onka, Katarzyna Zaj#c, Joanna Dracz, Kamila Pytel, #ukasz Mateuszuk, Stefan Ch#opicki. 2008. Platelets augment respiratory burst in neutrophils activated by selected species of gram-positive or gram-negative bacteria. *Folia Histochemica et Cytobiologica* **46**:3, 383-388. [CrossRef]
12. J. Stefanska, R. Pawliczak. 2008. Apocynin: Molecular Aptitudes. *Mediators of Inflammation* **2008**, 1-10. [CrossRef]
13. Grace Y. Sun, Lloyd A. Horrocks, Akhlaq A. Farooqui. 2007. The roles of NADPH oxidase and phospholipases A 2 in oxidative and inflammatory responses in neurodegenerative diseases. *Journal of Neurochemistry*, ahead of print070611013409004-???. [CrossRef]
14. Shane Miersch, Inga Sliskovic, Arun Raturi, Bulent Mutus. 2007. Antioxidant and antiplatelet effects of rosuvastatin in a hamster model of prediabetes. *Free Radical Biology and Medicine* **42**:2, 270-279. [CrossRef]
15. ANGEL COGOLLUDO, GIOVANNA FRAZZIANO, LAURA COBEÑO, LAURA MORENO, FEDERICA LODI, EDUARDO VILLAMOR, JUAN TAMARGO, FRANCISCO PEREZ-VIZCAINO. 2006. Role of Reactive Oxygen Species in Kv Channel Inhibition and Vasoconstriction Induced by TP Receptor Activation in Rat Pulmonary Arteries. *Annals of the New York Academy of Sciences* **1091**:1, 41-51. [CrossRef]
16. A BEGONJA, L TEICHMANN, J GEIGER, S GAMBARYAN, U WALTER. 2006. Platelet regulation by NO/cGMP signaling and NAD(P)H oxidase-generated ROS. *Blood Cells, Molecules, and Diseases* **36**:2, 166-170. [CrossRef]
17. Agnes Görlach . 2005. Redox Regulation of the Coagulation Cascade. *Antioxidants & Redox Signaling* **7**:9-10, 1398-1404. [Abstract] [Full Text PDF] [Full Text PDF with Links]
18. S. Pillai, C. Oresajo, J. Hayward. 2005. Ultraviolet radiation and skin aging: roles of reactive oxygen species, inflammation and protease activation, and strategies for prevention of inflammation-induced matrix degradation - a review. *International Journal of Cosmetic Science* **27**:1, 17-34. [CrossRef]
19. Agnes Görlach . 2004. Redox Control of Blood Coagulation. *Antioxidants & Redox Signaling* **6**:4, 687-690. [Citation] [Full Text PDF] [Full Text PDF with Links]