In Vitro Susceptibility of *Wistar* Rat Platelets to Hydrogen Peroxide and AAPH-Induced Oxidative Stress

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Abstract Hydroxyl and peroxy radicals are biologically active species because of their likelihood to damage cellular constituents. An in vitro study on *Wistar* rats was conducted to investigate the influence of hydrogen peroxide (H_2O_2) and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) on platelets and compare the vulnerability of platelets to oxidative stress (OS) induced by these two free radical initiators. Isolated platelets were divided into controls (without free radical initiators; $n = 5$) and experimentals (with free radical initiators; $n = 5$). Different concentrations (0.5, 1.0 and 2.0) of free radical initiators H_2O_2 and AAPH were used to treat the platelets and incubated for 5, 15 and 30 min. Biomarkers such as platelet aggregation, superoxide generation, lipid peroxidation (thiobarbituric acid reactive substances, conjugate dienes), protein oxidation (protein carbonyls, sulfhydryls) and antioxidant enzymes were assessed. In H_2O_2 and AAPH treated platelets, though OS was observed at concentrations of 0.5 and 1.0 mM, platelets could tolerate the oxidative insult. Treatment of platelets with 2.0 mM H_2O_2 demonstrated the onset of irreversible changes in platelets as observed in the results of increased superoxide generation and lipid peroxidation products. In 2.0 mM AAPH platelets, the oxidative damage was evident as indicated through increased aggregation, superoxide generation and conjugate dienes and lower protein sulfhydryls. Platelets were more susceptible to AAPH than H_2O_2 , as AAPH acted on both lipids and proteins whereas H_2O_2

acted only on lipids. This study gives insight on platelet survival under different OS situations.

Keywords 2,2'-Azobis(2-amidinopropane) dihydrochloride · Antioxidant enzymes · Hydrogen peroxide · Lipid peroxidation · Platelets · Protein oxidation

Introduction

Redox changes occur as a function of normal platelet activation, but the introduction of additional oxidative stress (OS) in certain situations may be prothrombotic. Reactive oxygen species (ROS) derived from both platelets and other vascular sources alter platelet responses. Platelet derived ROS can be involved in the regulation of platelet activation. Superoxide and hydroperoxy derivatives of long chain fatty acids are produced by platelets. Superoxide is known to enhance platelet aggregation. Low concentrations ($\mu\text{M/L}$) of hydrogen peroxide (H_2O_2) inhibit platelet function, although high concentrations (mM/L) of H_2O_2 have been shown to stimulate platelet aggregation [1–3].

H_2O_2 is a strong physiological oxidant which induces OS in platelets. The toxic effects of H_2O_2 may be associated with damage to different cellular components and changes in platelet functions. It easily penetrates cell membranes, transforms into more active forms and can independently cause or exacerbate platelet aggregation [4].

2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) is often used as a source of free radicals, which are formed in hydrophilic compartments. It decomposes to give free carbon-centred radicals, which react with oxygen quite rapidly to yield peroxy radicals [5, 6].

In several cardiovascular diseases (CVDs), such as atherosclerosis, diabetes mellitus and hypertension, intracellular

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redox homeostasis is disturbed, as a result of drastic increase in ROS levels that overwhelms the intrinsic antioxidant defence mechanisms. This situation is known as “Oxidative Stress” (OS) and is associated with significant damage of cellular molecules, their dysfunction and alteration of cell structure. Platelet derived ROS supplement the circulating OS and are prothrombotic in various situations [7].

It has been found that OS in platelets leads to thrombocytosis, usually detected during the progression of Kawasaki disease (KD) [8]. The OS in blood influence platelet reactivity and platelet activating factor (PAF)—a family of lipids that are associated with the persistence of depressive episode and related neurodegenerative pathology in coronary artery disease (CAD) [9]. Anorexia nervosa, associated with high cardiovascular mortality is related to reduced L-arginine transport, NOS activity and cGMP basal levels in platelets due to increased OS [10]. Fibrinogen (Factor X) is one of the plasma proteins most susceptible to oxidative modification, which may cause thrombotic/bleeding complications associated with many pathophysiological states [11]. Hence, platelet OS and intra-platelet redox control are basic research subjects of relevance for understanding the involvement of platelets in atherogenic, inflammatory and antigenic processes, beyond their crucial role in haemostasis and thrombosis.

There are few reports on free radical initiators and platelets. Studies on H_2O_2 have focused mainly on platelet aggregation and glutathione system [4, 12–14]. Generation of peroxyl radicals is common to all organisms and may be high under OS [15]. AAPH studies in platelets have investigated on lipids peroxidation and aggregation [16–18].

The role of H_2O_2 and AAPH on platelet survival under OS and their response to different concentrations of these free radical initiators is unclear. A comparative study on H_2O_2 and AAPH in platelets has not been reported earlier. Thus, the present study was conducted to investigate the influence of H_2O_2 and AAPH on platelets and compare the vulnerability of platelets to these two free radical initiators. The following markers were analyzed towards the above objectives: (i) platelet integrity through aggregation and (ii) OS through superoxide generation, lipid peroxidation (thiobarbituric acid reactive substances (TBARS) and conjugate dienes), protein oxidation (protein carbonyls and sulfhydryls) and antioxidant enzymes—superoxide dismutase (SOD) and catalase (CAT).

Experimental Procedures

Animal care and maintenance was in accordance with the ethical committee regulations.

Table 1 Experimental design

Groups	Controls ($n = 5$)		Experimentals ($n = 5$)	
	Platelets in Tyrode's buffer		Platelets in Tyrode's buffer + free radical initiators	
Free radical initiators	–		H_2O_2	AAPH
Concentrations of free radical initiators (mM)	–		0.5, 1.0, 2.0	0.5, 1.0, 2.0
Incubation period (min)	–		5, 15, 30	15, 30

Chemicals

AAPH and cytochrome C were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other chemicals were of reagent grade and organic solvents of spectral grade.

Blood Sampling

According to Vani et al. [19] 4 months old male *Wistar* rats were used for sampling of blood. Blood was aspirated carefully from the heart into tubes with citrate, phosphate, dextrose and adenine (CPDA-1) solution.

Experimental Design

OS was induced in platelet suspension by H_2O_2 and AAPH as described in Table 1.

Isolation of Platelets

Platelets were isolated from the blood collected from five *Wistar* rats for each biomarker. Blood was centrifuged at $190 \times g$ for 15 min at room temperature to obtain platelet-rich-plasma (PRP). The PRP was then centrifuged at $2,500 \times g$ for 15 min at 22 °C. The resulting platelet pellet was gently resuspended in Tyrode's buffer, pH 7.4 [20].

Platelet Aggregation

100 μ l of platelet sample was added to each well of the microtitre plate and incubated at 37 °C. The absorbance was recorded at 405 nm in shaking mode for 1.5–2 min [21].

Superoxide Generation

Superoxide generation in platelets was measured by Cytochrome C (Cyt C) reduction according to Olas and

Wachowicz [22]. 200 μl Cyt *C* (160 μM) was added to the platelets and incubated at 37 °C. The samples were centrifuged at 3,500 rpm for 5 min. Reduction of Cyt *C* was measured spectrophotometrically at 550 nm. To calculate the molar concentration of superoxide, extinction coefficient for Cyt *C* of 18,700 $\text{M}^{-1} \text{cm}^{-1}$ was used.

Thiobarbituric Acid Reactive Substances (TBARS)

TBARS was measured according to Olas et al. [23]. The platelet samples were cooled in an ice bath for 10 min. The samples were then transferred to an equal volume of 20 % (v/v) cold trichloroacetic acid (TCA) in 0.6 M HCl and centrifuged at 1,200 $\times g$ for 15 min. The supernatant was mixed with 0.2 ml of 0.12 M thiobarbituric acid (TBA) in 0.26 M Tris at pH 7.0. The mixture was incubated in a boiling water bath for 15 min and the absorbance was read at 532 nm.

Conjugate Dienes

Conjugate dienes was measured according to Olas and Wachowicz [22]. Platelet samples were transferred to ether/ethanol, 1:3 (v/v) mixture and vortexed. The mixture was centrifuged at 6,000 $\times g$. The level of conjugate dienes was measured spectrophotometrically at 235 nm.

Protein Carbonyls

Protein carbonyls were measured according to Reznick and Packer [24]. Platelet samples were mixed with 2 ml of 10 mM dinitrophenyl hydrazine (DNPH) in 2.5 M HCl and incubated for 1 h at room temperature. After incubation, 2.5 ml of 20 % TCA was added and left in ice for 10 min. Mixture was centrifuged at 3,000 rpm and supernatant was discarded. The protein pellets were washed three times with ethanol:ethyl acetate (1:1 (v/v)). The final precipitate was dissolved in 1 ml of 6 M guanidine HCl in 133 mM Tris. The absorbance was read at 355–390 nm. The carbonyl content was calculated using absorption coefficient of 22,000 $\text{M}^{-1} \text{cm}^{-1}$.

Protein Sulfhydryls

The protein sulfhydryls were measured according to Habeeb [25]. 1.5 ml of pH 8.0 buffer (0.08 mol l^{-1} Na- PO_4 , 0.5 mg ml^{-1} of Na_2 -EDTA and 2 % SDS) was added to the platelet samples. Mixture was vortexed and then 0.1 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was added. This was incubated at room temperature for 15 min. Absorbance was read at 412 nm. Molar absorptivity of 13,600 $\text{M}^{-1} \text{cm}^{-1}$ was used to calculate protein sulfhydryls.

Superoxide Dismutase [SOD, 1.15.1.1]

SOD in the platelet sample was measured according to Misra and Fridovich [26]. Platelet samples were added to 880 μl of carbonate buffer (0.05 M, pH 10.2, 0.1 mM EDTA). Epinephrine (20 μl of 30 mM in 0.05 % acetic acid) was added to the mixture and absorbance was measured at 480 nm for 4 min. SOD activity was expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50 % which is equal to 1 unit.

Catalase [CAT, 1.11.1.6]

Catalase was measured according to Aebi [27]. To 100 μl of platelet sample, 10 μl of absolute ethanol was added and incubated in ice bath for 30 min. After incubation, 240 μl of phosphate buffer was added to the above sample. 250 μl of 0.066 M H_2O_2 was added just before reading and absorbance was measured at 240 nm. The molar extinction coefficient of 43.6 $\text{M} \text{cm}^{-1}$ was used to determine the catalase activity. 1 unit of activity is equivalent to mol H_2O_2 degraded $\text{min}^{-1} \text{mg}^{-1}$ protein.

Statistical Analyses

Results are represented as mean $\pm$ SE. Two-way ANOVA was performed between the groups and sub-groups and the results are considered significant at $P < 0.05$. Bonferroni *Post Test* was performed using GraphPad Prism 6 Software.

Results

Aggregation

Aggregation increased in 1.0 mM H_2O_2 platelets and decreased in 2.0 mM H_2O_2 platelets when compared with control (Fig. 1a). Aggregation in AAPH platelets increased with AAPH concentration (Fig. 1b).

Superoxide

Superoxide significantly increased in H_2O_2 treated platelets with respect to control. One fold increase was noticed in 1.0 mM H_2O_2 (5 min) platelets with respect to control (Fig. 2a). Superoxide in AAPH treated platelets demonstrated insignificant variation with respect to control (Fig. 2b).

TBARS

TBARS increased in H_2O_2 treated platelets when compared with control. 1.0 mM H_2O_2 (30 min) and 2.0 mM H_2O_2

Fig. 1 a Aggregation in platelets treated with H_2O_2 . 5, 15 and 30 min; incubation of platelets with different concentrations of H_2O_2 . **b** Aggregation in platelets treated with AAPH. 15 and 30 min; incubation of platelets with different concentrations of AAPH. Values are mean $\pm$ SE of five animals/group. Two way ANOVA was performed between the groups and sub-groups to analyze aggregation followed by Bonferroni *Post* test, using GraphPad Prism software. $P < 0.05$ was considered significant. Values between groups are represented in *upper case*. Changes within the groups are represented in *lower case*. Those not sharing the same letters are significantly different

(15 min) platelets demonstrated threefold elevation in TBARS when compared with control. TBARS increased in AAPH platelets with respect to control (Table 2).

Conjugate Dienes

Conjugate dienes increased in H_2O_2 platelets with respect to control. Increments of fivefold (15 min) and sixfold (30 min) in 1.0 mM AAPH and tenfold (15 min) and 11 fold (30 min) in 2.0 mM AAPH was observed when compared with control (Table 2).

Protein Carbonyls

Protein carbonyls decreased in H_2O_2 platelets but an elevation of twofolds was observed in 0.5 mM H_2O_2 (5 min) platelets against control. Protein carbonyls reduced in AAPH treated platelets when compared with control (Table 2).

Protein Sulfhydryls

Protein sulfhydryls varied insignificantly in H_2O_2 treated platelets with respect to control. Protein sulfhydryls reduced in AAPH platelets when compared with control (Table 2).

Superoxide Dismutase

SOD increased significantly in H_2O_2 and AAPH platelets against control (Table 2).

Catalase

H_2O_2 treated platelets showed insignificant variation in catalase activity with control and different concentrations of H_2O_2 . Catalase increased by twofolds in 1.0 mM AAPH (30 min) platelets when compared with control and 0.5 mM AAPH (15 min).

Fig. 2 a Superoxide generation in platelets treated with H_2O_2 . 5, 15 and 30 min: incubation of platelets with different concentrations of H_2O_2 . **b** Superoxide generation in Platelets treated with AAPH. 15 and 30 min: incubation of platelets with different concentrations of AAPH. Values are mean $\pm$ SE of five animals/group. Two way ANOVA was performed between the groups and sub-groups to analyze superoxide generation followed by Bonferroni *Post* Test, using GraphPad Prism software. $P < 0.05$ was considered significant. Values between groups are represented in *upper case*. Changes within the groups are represented in *lower case*. Those not sharing the same letters are significantly different

Discussion

Aggregation was insignificant in 0.5 mM H_2O_2 samples, which was in accordance with the results of Canoso et al. [28]. Aggregation increased in platelets treated with 1.0 mM H_2O_2 than in 0.5 mM H_2O_2 platelets due to increase in free radical generation that can potentiate platelet aggregation [29]. 2–10 mM H_2O_2 is reported to damage the structure of platelet membrane and inhibit ADP-induced platelet aggregation [30, 31], as evident from our result of decreased aggregation in 2 mM H_2O_2 platelets.

Aggregation of AAPH treated platelets increased proportionately with concentration. This can be due to elevated Ca^{2+} mobilization and ATP release by the enzymes lipoyxygenase and cyclooxygenase, that increase platelet aggregation [32, 33].

Superoxide generation in H_2O_2 treated platelets increased with concentration when compared with control. Superoxides have overwhelmed the platelet SOD. The efficacy of superoxide dismutation into H_2O_2 is affected by the abundance of intracellular SOD [34].

Superoxide generation was insignificant in AAPH treated platelets, as the peroxy radicals generated from AAPH do not dissociate to superoxide radicals due to the stability of carbon–oxygen bond [35, 36].

TBARS increased in H_2O_2 treated platelets with respect to control either due to increased hydroxyls which can abstract hydrogen atom in PUFA and initiate lipid peroxidation [37] or inadequate antioxidants [38].

TBARS increased in 0.5 mM AAPH platelets and decreased in 1.0 and 2.0 mM AAPH platelets when compared with control. This may be due to the elevation of peroxy radicals that initiate free radical chain reactions

Table 2 Lipid peroxidation, protein oxidation and superoxide dismutase in platelets treated with H₂O₂ and AAPH

Conc (mM)	Exposure time (min)	TBARS* (μmol/mg protein)	Conjugate Dienes* (μmol/mg protein)	Protein carbonyls* (μmol/mg protein)	Protein sulfhydryls [#] (μmol/mg protein)	SOD* (U/mg protein)
Con	–	3.86 ± 1.2	26.48 ± 3.8	396.31 ± 98.1	251.45 ± 48.0	27.99 ± 5.0
H ₂ O ₂						
0.5	5	2.95 ± 0.8 ^a	17.72 ± 7.4 ^a	1,279.17 ± 313.9 ^a	232.12 ± 47.2 ^a	11.02 ± 2.1 ^a
	15	23.34 ± 2.5 ^b	28.79 ± 11.2 ^a	13.0 ± 4.5 ^b	344.25 ± 60.2 ^a	59.08 ± 9.3 ^b
	30	7.60 ± 1.0 ^a	103.50 ± 20.7 ^b	28.10 ± 5.9 ^b	227.17 ± 55.8 ^a	65.79 ± 10.2 ^b
1.0	5	2.77 ± 1.7 ^a	80.28 ± 12.8 ^a	36.96 ± 15.2 ^a	271.38 ± 159.1 ^a	121.21 ± 23.1 ^a
	15	13.62 ± 2.2 ^b	68.58 ± 8.8 ^a	48.76 ± 11.81 ^a	144.76 ± 39.1 ^a	62.15 ± 11.9 ^b
	30	16.26 ± 4.1 ^b	52.59 ± 22.2 ^a	79.26 ± 10.2 ^a	257.50 ± 77.6 ^a	65.65 ± 8.1 ^b
2.0	5	1.30 ± 0.1 ^a	21.46 ± 7.9 ^a	500.22 ± 169.1 ^a	88.01 ± 13.4 ^a	22.35 ± 2.6 ^a
	15	17.41 ± 2.6 ^b	60.39 ± 14.1 ^{a,c}	34.94 ± 10.1 ^b	297.51 ± 49.5 ^a	58.33 ± 5.3 ^b
	30	6.09 ± 1.4 ^a	68.48 ± 12.7 ^{b,c}	19.17 ± 1.4 ^b	129.62 ± 51.5 ^a	69.15 ± 9.8 ^b
AAPH						
0.5	15	6.45 ± 1.4 ^a	93.58 ± 16.3 ^a	25.79 ± 8.8 ^a	158.30 ± 23.5 ^a	46.73 ± 8.7 ^a
	30	4.10 ± 1.4 ^a	131.52 ± 20.9 ^a	37.64 ± 4.5 ^a	201.65 ± 43.7 ^a	45.07 ± 8.6 ^a
1.0	15	1.18 ± 0.5 ^a	167.32 ± 20.3 ^a	3.66 ± 3.5 ^a	127.84 ± 22.1 ^a	41.79 ± 8.4 ^a
	30	2.20 ± 0.5 ^a	203.94 ± 21.4 ^a	11.94 ± 2.1 ^a	130.65 ± 22.3 ^a	41.03 ± 4.1 ^a
2.0	15	3.30 ± 0.6 ^a	305.45 ± 40.1 ^a	9.63 ± 4.5 ^a	133.75 ± 23.2 ^a	39.69 ± 3.5 ^a
	30	2.21 ± 1.4 ^a	323.02 ± 37.9 ^a	13.69 ± 1.4 ^a	188.21 ± 42.4 ^a	43.53 ± 3.6 ^a

Values are expressed as mean ± SE of five animals/group. Changes between groups and sub-groups were analysed by two way ANOVA followed by Bonferroni *Post Test*, using GraphPad Prism software. *P* < 0.05 was considered significant

Conc concentration of free radical initiators; Con control

* Significant changes were observed between the groups in TBARS, Conjugate dienes, Protein carbonyls and SOD of H₂O₂ and AAPH treated platelets

[#] Significant changes were observed between the groups in protein sulfhydryls of AAPH treated platelets

^{a, b, c} Changes within the groups are represented in *lower case*. Those not sharing the same letters are significantly different

[17, 39]. 1.0 and 2.0 mM AAPH could be the optimum levels required for activation of antioxidant system (hormesis effect), thereby resulting in decreased TBARS [40].

Conjugate dienes elevated in H₂O₂ platelets with respect to control. The maximum increase observed at 30 min can be due to the lag period caused by the action of antioxidants before the onset of detectable lipid peroxidation [37]. The primary products of lipid peroxidation (conjugate dienes) increased, while the secondary products (TBARS) decreased at 30 min incubation, due to action of platelet antioxidants.

Conjugate dienes increased with concentration and time in AAPH treated platelets. Cells exposed to OS by peroxyl radical generators show elevated levels of oxidation as indicated by lipid peroxidation [41].

Protein carbonyls decreased in H₂O₂ and AAPH platelets when compared with control indicating that platelet antioxidants could protect the proteins from oxidative modification [23, 40].

Protein sulfhydryls were insignificant in H₂O₂ treated platelets. This can be due to the intracellular glutathione pool which protects the protein sulfhydryls from irreversible oxidation [42].

A decrement in protein sulfhydryls of AAPH platelets against control elucidates the generation of peroxyl radicals by AAPH thereby oxidising sulfhydryls to disulphides [43].

SOD increased in H₂O₂ and AAPH platelets with respect to control. Elevation in SOD was in accordance with the superoxide generation. The level of superoxides regulate the rate of SOD activity [39], which is also evident from our study.

Catalase was insignificant in H₂O₂ platelets which can be due to (i) hydroperoxides produced by the platelets are also metabolized by GSH-Px present in the platelets, at lower concentrations of H₂O₂ [3, 44] and (ii) H₂O₂ at concentrations other than 0.025, 0.05 and 0.075 mM decrease CAT activity [45].

AAPH triggers the formation of H₂O₂ in addition to the endogenous H₂O₂ [3], also observed in our results of 1.0 mM AAPH (30 min). This is corroborated through reduced TBARS.

In conclusion, though H₂O₂ and AAPH induced OS at concentrations of 0.5 and 1.0 mM; platelets were not impaired. Treatment of platelets with 2.0 mM H₂O₂ and AAPH demonstrated the onset of irreversible changes in platelets. But platelets were more susceptible to AAPH

than H_2O_2 , as AAPH acted on both lipids and proteins whereas H_2O_2 acted only on lipids.

We have elucidated the response of platelets to OS induced by two different free radical initiators. This is the first study that gives a comprehensive view about the susceptibility of platelets to different concentrations of the two important free radicals produced under physiological conditions, i.e. hydroxyl and peroxy radicals. Hence it paves way towards (i) better management of diseases (such as cardiovascular diseases and various platelet disorders) arising due to ROS and; (ii) improving the efficacy of stored platelets and thereby prolonging their shelf-life.

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Conflict of interest The authors report no conflicts of interest.

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