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Adiponectin inhibits hyperlipidemia-induced platelets aggregation via attenuating oxidative/nitrative stress

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Summary

Adiponectin acts as an endogenous antithrombotic factor. However, the mechanisms underlying the inhibition of platelet aggregation by adiponectin still remain elusive. The present study was designed to test whether adiponectin inhibits platelet aggregation via attenuating oxidative/nitrative stress. Adult rats were fed a regular or high-fat diet for 14 weeks. The platelet was immediately separated and stimulated with recombinant full-length adiponectin (rAPN) or not. The platelet aggregation, nitric oxide (NO) and superoxide production, endothelial nitric oxide synthase (eNOS)/inducible NOS (iNOS) expression, and antioxidant capacity were determined. Treatment with rAPN inhibited hyperlipidemia-induced platelets aggregation ($P<0.05$). Interestingly, total NO, a crucial molecule depressing platelet aggregate and thrombus formation，was significantly reduced, rather than increased in rAPN-treated platelets. Treatment with rAPN markedly decreased superoxide production (-62%, $P<0.05$) and enhanced antioxidant capacity (+38%, $P<0.05$) in hyperlipidemic platelets. Noticeably, hyperlipidemia-induced reduced eNOS phosphorylation and increased iNOS expression was significantly reversed following rAPN treatment ($P<0.05$, $P<0.01$, respectively). Taken together, these data suggest that adiponectin is an adipokine that suppresses platelet aggregation by enhancing eNOS activation and attenuating oxidative/nitrative stress including blocking iNOS expression and superoxide production.

Keywords: Adiponectin; Platelet; Aggregation; Hyperlipidemia

1. Introduction

Metabolic syndrome (MS) is characterized by a series of metabolic and hemodynamics alterations, which increase the incident of endothelial damage and atherosclerosis, becoming the leading cause of cardiovascular disease.(Hulthe et al. 2000; Sakkinen et al. 2000) Of note, hyperlipidemia, the critical composition of metabolic syndrome, is closely related to atherosclerosis and formation of thrombus. Dyslipidemia is accompanied by platelet hyperactivity, hypercoagulability with increased factor VII, and hypofibrinolysis with increased plasminogen activator inhibitor (PAI)-1.(Juhan-Vague & Vague 1990) The increased flux of nonesterified fatty acids (NEFAs) from the adipocytes increases tissue factor (TF) and PAI-1 levels and enhances platelet aggregation; All of these obviously promote thrombosis.(Eckel et al. 2002)

Adiponectin is a cytokine predominantly secreted from adipose tissue and abundantly presents in plasma. Numerous studies have shown that plasma levels of adiponectin decrease in obesity, type 2 diabetes, and patients with coronary artery disease (CAD)(Hotta et al. 2000; Matsuzawa et al. 2004). In adiponectin knockout (APN-KO) male mice, an accelerated thrombus formation has been observed. Adenovirus-mediated supplementation of adiponectin attenuates the enhanced thrombus formation.(Kato et al. 2006) Decreased adiponectin serum levels in patients with essential hypertension correlate well with changes in ADP-induced conventional platelet aggregation(Ekmekci et al. 2008), this association may potentially contribute to future thrombus formation and higher risks for cardiovascular events in those patients. These data suggest that adiponectin acts as an endogenous antithrombotic factor. However, the mechanisms underlying the inhibition of platelet aggregation by adiponectin still remain largely elusive.

Platelet activation occurs through the stimulation of a large number of exquisitely integrated positive signaling pathways that ensure rapid activation at sites of vascular injury.

Platelets may also generate nitric oxide (NO), although the presence of NO synthase (NOS) and the role of platelet-derived NO are both controversial.(Naseem & Riba 2008) It was suggested that platelet-derived NO depress platelet aggregate and thrombus formation.(Freedman et al. 1997; Freedman et al. 1999) At the same time, platelet-derived NO has been suggested to be a critical component of the platelet activation pathway in response to von Willebrand factor (VWF) and thrombin.(Li et al. 2003; Li et al. 2006) Regardless of the role of NO, all of these studies agree that NO mediates platelet actions through the stimulation of sGC and downstream activation of PKG, although they may be critical for the interpretation of the potential role of this related enzyme in platelets. Furthermore, to the best of our knowledge, the possibility that adiponectin may play a crucial role in protection of vasculature from hyperlipidemia-promoted platelet aggregation and thrombosis formation has never been studied.

In this study, we have provided the first evidence that as an antithrombotic factor, adiponectin inhibits hyperlipidemia-induced platelet aggregation via eNOS/NO pathway.

2. Material and methods

Animals

All the experimental procedures were in accordance with the National Institutes of Health guidelines and were approved by the local authorities for animal research. Twenty male Sprague-Dawley rats (8 wk old) were randomized to receive a regular chow diet or a high-fat supplemented diet (detailed composition: 1% cholesterol, 10% lard, 10% yolk powder, 0.2% porcine cholate, 78.8% regular chow). Food and water were provided ad libitum, and animals were maintained in a temperature-controlled barrier facility with a 12:12-h light-dark cycle. Fourteen weeks later, animals were anesthetized by intraperitoneal administration of 20% urethane. Caval blood was withdrawn, the platelet was immediately separated, and lipid profile, glucose, and insulin levels were determined as described below.

Plasma lipid determinations

Plasma cholesterol and triglyceride levels were determined by a biochemistry analyzer (Cobas Integra 400 Plus, Roche) in accordance with the manufacturer's instructions.

Isolation of rat platelets

Rat platelets were isolated as the method described previously(Riba et al. 2008). Briefly, caval blood was taken using acid citrate dextrose (ACD: 29.9 mM sodium citrate, 113.8 mM glucose, 72.6 mM NaCl and 2.9 mM citric acid, pH 6.4) as anticoagulant. Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200g×20 min. Washed platelets (WP) were isolated from PRP by centrifugation at 800g×12 min in the presence of prostaglandin E1 (PGE1; 50 ng/mL). The platelet pellet was resuspended at a concentration of 2×10^8 platelets/mL in modified Tyrodes buffer (150 mM NaCl, 5 mM HEPES, 0.55 mM NaH_2PO_4, 7mM $NaHCO_3$, 2.7 mM KCl, 0.5 mM $MgCl_2$ and 5.6 mM glucose, pH 7.4).

Platelet aggregation

Platelet aggregation was analyzed by a improved turbidimetric method(Born 1962). Tyrode solution was used to adjust the baseline of minimal light transmission. Platelet aggregation was recorded under constant stirring conditions (900 rpm×6 min) in optical density (OD) by platelet aggregation system (Shanghai Record Instrument, China). In brief, rat WP (2×10^8/mL) were placed in 20-well cell culture plates, stimulated at 37℃ with vehicle or 10 μg/mL recombinant full-length adiponectin (rAPN, BioVendor Laboratory Medicine, Czech Republic) for 1 hour, the fall in absorbance of WP being determined 1-5 min after addition of a standard amount of aggregating agent thrombin (1 IU/mL). A portion of rAPN-treated platelets were pre-incubated with N^G-Monomethyl-L-arginine (L-NMMA; 1 mM) for 10 min. Each treatment group contains 5 wells of platelets.

The percentage of aggregation was calculated by the formula, where absorbance is measured by optical density (OD): % aggregation={[OD before the addition of thrombin－OD after the addition of thrombin]/[OD before the addition of thrombin－OD of Tyrode solution]}×100. All studies were performed at least twice on two separate occasions in triplicate, and data with standard deviations within 10% of the mean are reported(Chia et al. 2004).

Platelet aggregation was also examined by using a fluorescence microscope and followed immunofluorescence detection procedures described previously(Karpman et al. 2001). Treated platelets were fixed with 1% paraformaldehyde at 25°C for 30 min. After fixation, platelets were seed onto glass slides. Coated glass slides were washed three times with PBS, and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat CD9 monoclonal antibody was added and incubated at 37°C for 1 h. Slides were washed four times in PBS, and covered with fluorescent mounting gel (Biomeda, Vancouver, Canada) before being examined under an immunofluorescence microscope. For each slide, 10 fields were randomly chosen and using a defined rectangular field area (20 × objective), a total of 100 platelets per field were counted. The aggregation index was determined. The assays were performed in a blinded manner.

Total NO production measurement

Platelets total NO production was determined by measuring the concentration of nitrite, a stable metabolite of NO in vitro, with a modified Griess reaction method (Cosentino et al. 1997). Briefly, control platelets or hyperlipidemic platelets were stimulated with vehicle, rAPN (10 μg/mL), or rAPN addition of L-NMMA as above, 100 μl of culture medium was taken and mixed with an equal volume of modified Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). After 10 min of incubation at room temperature, the resultant chromophore was spectrophotometrically determined at 540 nm using a spectrophotometer (Spectra-Max 190, Molecular Device). The nitrite concentrations in the samples were calculated from freshly prepared nitrite standard curves made from sodium nitrite with the same medium.

Determination of eNOS and iNOS expression by Western blot

Platelets were sonicated in lysis buffer. After quantitation of protein content with Bradford protein assay, equal amounts of protein (40 μg protein/lane) were electrophoresed on a 14% SDS-polyacrylamide gel and electrophoretically transferred to a poly (vinylidene difluoride) membrane (Millipore, Billerica, MA). After blocking with 5% skim milk in Tris-buffered saline at room temperature for 1 h, the membrane was incubated with an antibody against eNOS, phosphorylated eNOS (Ser-1177), or iNOS (Cell Signaling Technology, Danvers, MA) overnight at 4°C. The membrane was then washed with PBS and incubated with horseradish peroxidase-conjugated IgG antibody (Cell Signaling) for 1 h at

37°C. The blots were developed with an enhanced chemiluminescence detection kit (Pierce Biotechnology, Rockford, IL). The immunoblotting was visualized with ChemiDocXRS (Bio-Rad Laboratory, Hercules, CA), and the blot densities were analyzed with LabImage software.

Determination of platelets antioxidant capacity

Platelets were sonicated in 0.9% NaCl solution (1:10, wt/vol), and centrifuged at 3,000 g for 5 min. The pellet was discarded. Total antioxidant capacity was determined with a spectrophotometric assay kit (Nanjing Jiancheng Bioengineering Institute), following the manufacture's instruction. In brief, 30 μl of supernatant was added to the reaction buffer containing xanthine, xanthine oxidase, and hydroxylamine. After 40 min of incubation at 37°C, accumulation of nitrite was quantified by the Griess reaction. Platelets antioxidant capacity is inversely related to the concentration of nitrate. Results were normalized against the mean value of control and expressed as fold changes.

Quantification of platelets superoxide production

Superoxide production from platelets was measured by flow injection chemiluminescence as described previously (Yao et al. 2004). Superoxide production was expressed as chemiluminescence intensity (CI) per microgram of protein (CI/μg protein).

Statistical analysis

Values are presented as means±SEM. Data were analyzed with one-way ANOVA (GraphPad Software, San Diego, CA). A probability value of $P < 0.05$ was considered to be statistically significant.

3. Results

Plasma lipid profile

Total cholesterol and triglyceride levels were found to be significantly higher in HF fed rats than those fed with control diet (2.0±0.1 vs 1.6±0.1 of Con, $P<0.01$ and 1.5±0.3 vs 1.1±0.3 of Con, $P<0.05$, respectively, n=8–10 animals/group.).

Treatment with rAPN inhibited hyperlipidemia-induced platelets aggregation

As illustrated in Fig. 1, hyperlipidemia caused a significant increase in platelets aggregation after 5 min treatment with thrombin ($P<0.01$). However, pre-treatment with rAPN for 1 h markedly reduced the hyperlipidemic platelets aggregation ($P<0.05$). To further determine whether rAPN inhibited thrombin-induced platelets aggregation by enhancing NO production, a portion of rAPN-treated platelets were pretreated with L-NMMA. Addition of 1mM L-NMMA prior to rAPN dramatically inhibited rAPN effects on platelets aggregation ($P<0.01$), Furthermore, fluorescence microscopy displayed an enhanced aggregation in hyperlipidemic platelets compared to control platelets ($P<0.05$, Fig. 2), treatment with rAPN made a moderate reduce in hyperlipidemic platelet aggregation ($P<0.05$). However, platelet aggregation significantly enhanced after addition of L-NMMA ($P<0.01$).

Treatment with rAPN decreased hyperlipidemia-induced NO overproduction

NO has been shown responsible for endothelial vasorelaxation and inhibition of platelet adhesion and aggregation(Riddell & Owen 1999). We attempted to obtain direct evidence that treatment with rAPN may increase NO production and thus inhibit hyperlipidemia-induced platelets aggregation. Surprisingly, although treatment of platelets with rAPN significantly decreased platelets aggregation, this treatment reduced, rather than increased, total NO production ($P<0.05$, Fig. 3). This paradoxical result suggests that more complex signaling mechanisms are involved in the protective effect of rAPN against hyperlipidemia-induced platelets aggregation.

Treatment with rAPN increased eNOS phosphorylation and reduced iNOS expression in hyperlipidemic platelets

Although adiponectin has been shown to stimulate NO production in cultured platelets by phosphorylating eNOS (Milward et al. 2006), treatment with rAPN did not increase but reduced total NO production in hyperlipidemic platelets (Fig. 3). These results suggest that the overall effect of rAPN on total NO production may involve a complex regulation of rAPN on different forms of NOS under hyperlipidemic conditions. To directly investigate this novel possibility, the effect of rAPN on eNOS phosphorylation and iNOS expression was determined. As summarized in Fig. 4, a significant reduction in eNOS phosphorylation ($P<0.05$) and a marked increase in iNOS expression ($P<0.01$) were observed in hyperlipidemic platelets. Pretreatment with rAPN almost completely normalized eNOS phosphorylation ($P<0.05$) and significantly reduced iNOS expression ($P<0.01$) in hyperlipidemic platelets. These results demonstrated that rAPN had opposite effects on p-eNOS and iNOS expression, and this differential regulatory role may explain the paradoxical finding that rAPN significantly inhibited platelets aggregation but reduced total NO production.

Treatment with rAPN significantly reduced superoxide overproduction and enhanced antioxidant capacity in hyperlipidemic platelets

The above-mentioned results demonstrated that overproduction of NO in hyperlipidemic platelets is due to overexpression of iNOS protein. However, increase in NO production failed to inhibit platelets aggregation, suggest that increased NO destruction may be responsible for hyperlipidemic platelets aggregation. In addition, our novel observation that rAPN inhibited platelets aggregation without increasing NO production indicates that rAPN may reduce platelets aggregation by preserving bioactive NO. To obtain direct evidence to support this hypothesis, additional experiments were performed. As summarized in Fig. 5A, a 2.5-fold increase in superoxide production was observed in platelets isolated from hyperlipidemic animals, and treatment with rAPN almost abolished (62% reduction compared with vehicle-treated platelets, *P*<0.05) the superoxide overproduction observed in hyperlipidemic platelets. Moreover, hyperlipidemia-induced reduction in total antioxidant capacity was significantly preserved after rAPN treatment (38% increase compared with vehicle-treated platelets, *P*<0.05, Fig. 5B).

4. Discussion

The present study yields several critical new observations. First, we have observed for the first time that acute treatment with rAPN significantly attenuated hyperlipidemia-induced platelets aggregation. Second, we have provided direct evidence that inhibiting superoxide production, suppressing iNOS-mediated NO overproduction and preserving NO from destruction are the major mechanisms by which adiponectin exerts its antithrombotic effect. This result not only provides additional evidence that reduced adiponectin in metabolic disorders contributes to the formation of thrombus, but raises the possibility that therapeutic application of rAPN may be a useful treatment of metabolic disorders with atherosclerosis.

The close correlation among obesity, the metabolic syndrome, and atherosclerosis are well established.(Cooper-DeHoff & Pepine 2007) However, mechanisms by which obesity promotes occurrence of thrombus are not well understood. Increasing attention has been paid to the direct antithrombotic effects of plasma proteins that originate from adipose tissue, especially adiponectin.(Goldstein & Scalia 2004) Decreased plasma adiponectin levels are observed in patients with diabetes, metabolic syndrome, and coronary artery disease.(Ouchi et al. 2006) Moreover, many studies in animal models and human subjects have demonstrated adiponectin is a critical anti-atherosclerosis molecule whose reduction may contribute to thrombus formation and platelets aggregation.(Kato et al. 2006; Ekmekci et al. 2009) The present study took a different approach and provided the first evidence that acute treatment with rAPN significantly attenuates platelets aggregation associated with hyperlipidemia. In comparison with our results, Riba et al.(Riba et al. 2008) identified recombinant globular domain of adiponectin (gAd), but not rAPN, stimulates platelet aggregation through tyrosine kinase-dependent signaling pathway. Different effects of various isoforms of adiponectin on platelet aggregation raise the possibility that they may have distinct receptors and activate specific pathway, respectively.

We have obtained several lines of evidence indicating that rAPN inhibits platelets aggregation by its novel antioxidative property. First, adiponectin significantly reversed the hyperlipidemia-induced reduction of antioxidant activity. Since either increased formation of antioxidant molecule or reduced expression of oxidant molecule can strengthen antioxidant activity, we secondly detected adiponectin effect on superoxide production. Consistent with the study by Sanguigni et al,(Sanguigni et al. 2002) increased superoxide was produced by hyperlipidemic platelets, and we further observed overexpression of superoxide, which was markedly suppressed by administration of adiponectin. It is well documented that superoxide reacts with NO at a near diffusion-limited rate, which is three times faster than the reaction between superoxide and superoxide dismutase.(Huie & Padmaja 1993) This reaction not only causes the inactivation of NO, a molecule depressing leucocyte adhesion and platelets aggregation, but also results in the formation of peroxynitrite anion ($ONOO^-$), a highly reactive and cytotoxic molecule.(Beckman & Koppenol 1996) Thus the superoxide/NO reaction is a "toxic switch" that plays a critic pathogenic role in the development of thrombus.

Platelets possess the L-arginine-NO pathway through constitutive NO synthase in humans.(Sase & Michel 1995) There is accumulating evidence to show that NO suppresses platelet aggregation and activation.(Gkaliagkousi et al. 2007) Previous studies in cultured endothelial cells or platelets have demonstrated that adiponectin activate eNOS and increase NO production.(Chen et al. 2003; Li et al. 2003) Surprisingly, our results indicated adiponectin treatment reduced NO production although enhanced eNOS activation. We finally found hyperlipidemia induced overexpression of iNOS, which has ability to produce excessive NO, and treatment with adipnoectin suppressed iNOS expression in hyperlipidemic platelets. Since under oxidative stress conditions, NO undergoes a rapid reaction with superoxide anions to form peroxynitrite, a toxic metabolite of NO, which could cause platelet dysfunction,(Redondo et al. 2005; Olas & Wachowicz 2007) it is conceivable that adiponectin inhibits platelet activation by suppressing the inactivation of NO and subsequent formation of peroxynitrite via its anti-oxidative properties.

In summary, the present study demonstrated for the first time that adiponectin is an adipokine that suppresses platelet aggregation and activation by enhancing eNOS activation and attenuates oxidative/nitrative stress by blocking iNOS expression and superoxide production. Loss of this dual-protective effect of adiponectin because of reduced adiponectin production and/or development of adiponectin resistance in patients with metabolic syndrome may play a critical pathogenic role in thrombus formation and atherosclerosis. If these effects was proved by treating the diet-fed rats with rAPN in vivo, This would significantly strengthen the present in vitro data.

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Disclosure

The authors declare that they have no conflict of interest.

Abbreviations

ACD	Acid citrate dextrose
APN-KO	Adiponectin knockout
CAD	Coronary artery disease
eNOS	Endothelial nitric oxide synthase
FITC	Fluorescein isothiocyanate
gAd	Globular domain of adiponectin
iNOS	Inducible nitric oxide synthase
L-NMMA	N^G-Monomethyl-L-arginine
MS	Metabolic syndrome
NEFAs	Nonesterified fatty acids
NO	Nitric oxide
OD	Optical density
$ONOO^-$	Peroxynitrite anion
PAI	Plasminogen activator inhibitor
PGE1	Prostaglandin E1
PKG	Proteinkinase G
PRP	Platelet-rich plasma
rAPN	Recombinant full-length adiponectin
sGC	Soluble guanylate cyclase
TF	Tissue factor
VWF	von Willebrand factor
WP	Washed platelets

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Figure legends

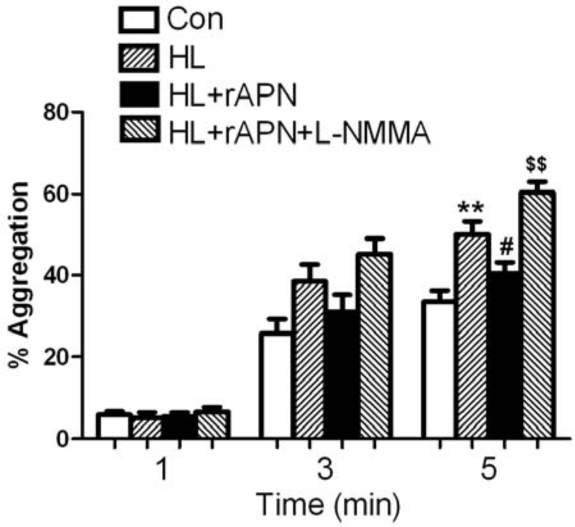
Fig. 1. Effect of rAPN on hyperlipidemia-induced platelets aggregation via turbidimetric method. Con, control; HL, hyperlipidemia; rAPN, recombinant full-length adiponectin; L-NMMA, N^G-Monomethyl-L-arginine. Values are means±SE; *n*=5 wells/group. $^{**}P<0.01$ vs. Con, $^{\#}P<0.05$ vs. HL. $^{\$\$}P<0.01$ vs. HL+rAPN.

Fig. 2. Effect of rAPN on hyperlipidemia-induced platelets aggregation using immunofluorescence detection. Representative fields of each slide were exhibited in control platelets (A), or hyperlipidemic platelets after treated with vehicle (B), rAPN (C) or rAPN addition of L-NMMA (D).

Fig. 3. Total nitric oxide production by platelets isolated from control (Con) or hyperlipidemic blood (HL) treated with vehicle, rAPN (HL+rAPN) or rAPN addition of L-NMMA (HL+rAPN+L-NMMA). NO concentration in medium containing platelets was determined by Griess reaction. Values are means±SE; *n*=6 wells of platelets/group. $^{**}P<0.01$ vs. Con. $^{\#}P<0.05$ vs. HL. $^{\$\$}P<0.01$ vs. HL+rAPN.

Fig. 4. Effect of rAPN on eNOS phosphorylation and iNOS expression in cultured platelets. A: expression of eNOS and phosphorylated eNOS (peNOS; at serine 1177, Western blots) and ratio of peNOS/eNOS density analysis. B: representative Western blots and density analysis for iNOS expression. Values are means±SE; *n*=6 wells of platelets/group. $^{*}P<0.05$ and $^{**}P<0.01$ vs. Con. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. hyperlipidemic platelets.

Fig. 5. Effect of rAPN on hyperlipidemia-induced superoxide overproduction (A) and reduction in antioxidant capacity (B). Values are means±SE; *n*=6 wells of platelets/group. $^{**}P<0.01$ vs. Con. $^{\#}P<0.05$ vs. hyperlipidemic platelets.


Con
HL
HL+rAPN
HL+rAPN+L-NMMA
80
60
40
20
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% Aggregation
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Time (min)

Fig. 1

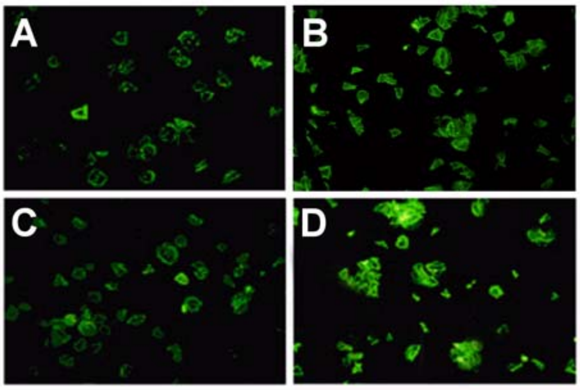

Fig. 2

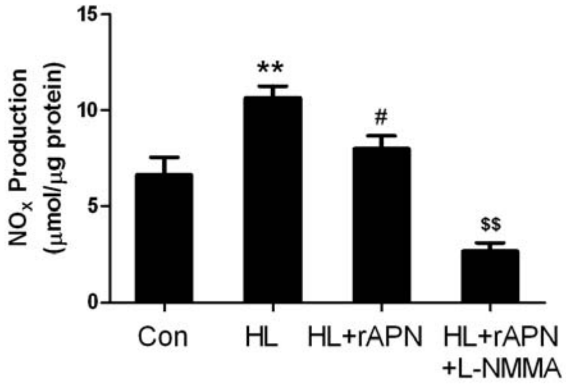
15
10
5
0
NOx Production
(μmol/μg protein)
**
#
$$
Con
HL
HL+rAPN
HL+rAPN
+L-NMMA

Fig. 3

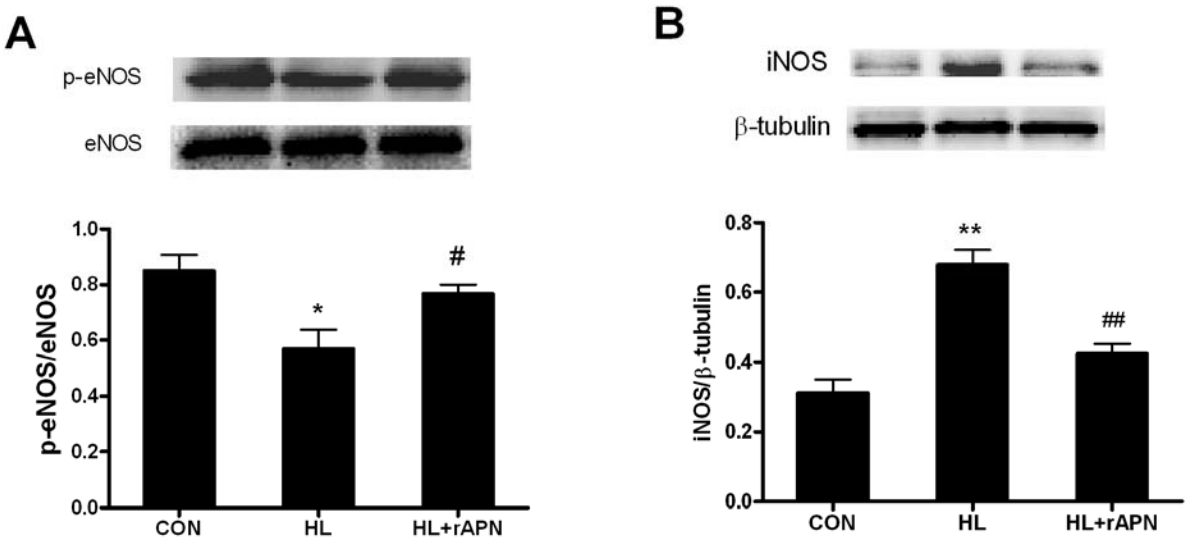
A
p-eNOS
eNOS
p-eNOS/eNOS
1.0
0.8
0.6
0.4
0.2
0.0
*
#
CON
HL
HL+rAPN
B
iNOS
β-tubulin
iNOS/β-tubulin
0.8
0.6
0.4
0.2
0.0
**
##
CON
HL
HL+rAPN

Fig. 4

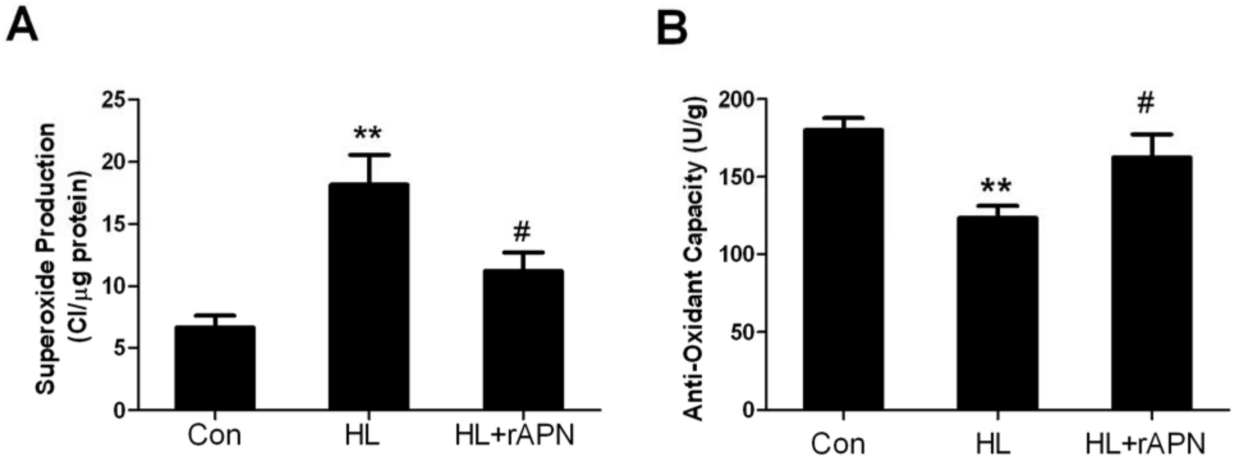
A
Superoxide Production (Cl/μg protein)
25
20
15
10
5
0
**
#
Con
HL
HL+rAPN
B
Anti-Oxidant Capacity (U/g)
200
150
100
50
0
**
#
Con
HL
HL+rAPN

Fig. 5