Periadventitial Adipose Tissue Promotes Endothelial Dysfunction via Oxidative Stress in Diet-Induced Obese C57Bl/6 Mice

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Background: Biological substances derived from perivascular fat modulate vascular tone, thus alterations in periadventitial adipose tissue (PVAT) may aggravate endothelial dysfunction in obesity.

Methods and Results: Male C57Bl/6 mice were fed either a high-fat diet or standard laboratory chow for 8 months. Vascular responses were studied in organ bath chambers from abdominal aortic ring preparations in the absence or presence of PVAT. The amount of PVAT as well as the cross-sectional area of adipocytes were increased in obese mice. In the presence of PVAT, obese aortas displayed impaired endothelium-dependent vasodilation whereas endothelium-independent vasodilation was unaltered. Endothelium-dependent vasodilation was restored after removal of PVAT and after reducing superoxide and hydrogen peroxide formation in the vascular wall by Tiron or polyethylene-glycol-catalase, respectively. PVAT from obese mice showed increased formation of hydrogen peroxide and superoxide. The PVAT-derived oxidative stress was abolished by pretreatment with the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase inhibitor, apocynin. The anti-contractile function of PVAT found in lean mice was completely abolished in obese mice, but partially restored after pretreatment with Tiron. The mRNA expressions of monocyte chemotactic protein-1, leptin and NADPH oxidase were markedly higher in the PVAT of obese than lean mice.

Conclusions: PVAT promotes endothelial dysfunction in diet-induced obese C57Bl/6 mice via mechanisms that are linked to increased NADPH oxidase-derived oxidative stress and increased production of pro-inflammatory cytokines. (Circ J 2010; 74: 1479–1487)

Key Words: Endothelial dysfunction; Obesity; Perivascular fat; Vasoconstriction

Recent experimental evidence has provided important evidence on the vasomodulatory function of periadventitial adipose tissue (PVAT), which has not been considered in earlier vascular function studies. PVAT, which surrounds most blood vessels, is now considered an active endocrine organ, generating and secreting a wide range of biological substances that critically influence vascular tone.1 PVAT attenuates the contractile responses to a variety of agonists in isolated large arteries obtained from mice, rats and humans.2–6 The attenuation of vasoconstriction depends on the amount of physiological adipose tissue and is based on continuous release of adipose tissue-derived relaxing factors, which enhance vasodilation via both endothelium-dependent and -independent mechanisms.4,5,7 In contrast, PVAT may enhance vascular contractile responses by preventing endothelium-dependent vasodilatation through inhibition of endothelial nitric oxide synthase.8 Furthermore, PVAT increases the extent of vasoconstriction via generation of contractile superoxide anions, which are released after electric field stimulation from functional reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase in rat mesenteric arteries.9 Lipoatrophic mice born without white fat tissue have a markedly decreased amount of PVAT accompanied with increased arterial pressure and compromised vascular function.10 Thus, PVAT plays an important role modulating vascular reactivity also in vivo."
the vascular wall.\textsuperscript{11-14} Obesity, however, is also characterized by the accumulation of ectopic fat in the heart, kidneys and vascular wall, which, in turn, is thought to increase the overall cardiovascular risk.\textsuperscript{15} There is accumulating experimental evidence to suggest that both structural and functional alterations in PVAT might contribute to the increased risk for atherosclerosis and hypertension through endothelial dysfunction.\textsuperscript{16-18} Accumulation of PVAT is associated with impaired anti-contractile function, perivascular inflammation via production of pro-inflammatory cytokines and chemokines and the accumulation of macrophages, as well as with dysregulation of adipocyte-derived hormones termed adipokines.\textsuperscript{19-22} In contrast, spontaneously hypertensive rats had reduced amounts of PVAT in resistance arteries with impaired anti-contractile function.\textsuperscript{23} These studies provide compelling evidence that PVAT plays an important role in the regulation of vascular tone. We used high-fat-fed C57Bl/6 male mice, which are a well-established model for diet-induced obesity, to test the hypothesis that alterations in PVAT play a role in the pathogenesis of endothelial dysfunction in obesity.

Methods

Animals and Diets

Three–four-week-old male C57Bl/6J mice were purchased from Harlan (Horst, The Netherlands). The mice were housed in groups of 5 per cage in a standard experimental animal laboratory, illuminated from 6:30 a.m. to 6:30 p.m., at a temperature of 22±1°C. The protocol was approved by the Animal Experimentation Committee of the Medical Faculty of University of Helsinki, Finland, whose standards correspond to those of the American Physiological Society. The mice had free access to feed and tap water during the experiment. After a 1-week acclimatization period, the mice were divided into 2 groups (both n=15) receiving either high-fat diet (Taconic D12492, protein 20%, carbohydrate 75% and fat 60% of energy; Research Diets, New Brunswick, NJ, USA) or standard laboratory animal diet (FG 811004, protein 17.5%, carbohydrate 75% and fat 7.5% of energy, Special Diets Services UK) for 8 months.

Body Weight and Fat Tissue Measurement

The body weight was monitored once a week and the consumption of feed was monitored daily using a standard laboratory table scale (Ohaus ScoutPro, SP4001, Nänikon, Switzerland). The amount of body fat was assessed on dual-energy X-ray absorptiometry (DEXA, Lunar PIXImus, GE Healthcare, Chalfont St Giles, UK) at the end of the study.

Oral Glucose Tolerance Test

At the end of the study the mice were weighed and glucose (2 mg/g body weight) was administered via an oral gavage (OGTT). Blood samples were drawn from the tail vein at 0 min, 15 min, 30 min, 60 min and 90 min after oral glucose exposure. Blood glucose (mmol/L) was analyzed with a glucometer (Super Glucocard II, GT-1630, Arkray Factory, Shiga, Japan).

Assessment of Vasomotor Function

After the treatment period, the mice were rendered unconscious with CO\textsubscript{2}/O\textsubscript{2} (95%\%/5%) (AGA, Riihimäki, Finland) and decapitated. Abdominal aortas were removed, quickly transferred to cold oxygenated Krebs solution, and dissected into 2–3-mm rings whereby perivascular fat and connective tissues were either removed or left intact. The aortic rings were studied using a conventional organ bath system (EMKA technologies, France 2000). Under a microscope the perivascular fat was removed with fine scissors, being careful not to damage the adventitia. Each aortic ring was positioned between 2 stainless steel wires (diameter 100 μm) in organ chambers filled with Krebs solution of the following composition: NaCl, 119.0 mmol/L; NaHCO\textsubscript{3}, 25.0 mmol/L; glucose, 11.1 mmol/L; CaCl\textsubscript{2}, 1.6 mmol/L; KCl, 4.7 mmol/L; KH\textsubscript{2}PO\textsubscript{4}, 1.2 mmol/L; MgSO\textsubscript{4}, 1.2 mmol/L (37°C, pH 7.4) and aerated continuously with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. Isometric tension was recorded continuously. In each pair, PVAT was left intact on 1 ring (PVAT+), whereas PVAT was removed from the other ring (PVAT–). After a 30-min equilibration period in the non-stretching condition, the pre-tension was stepwise increased to 0.4 g and it was allowed to rest over a period of 60 min. Then aortic rings were first challenged to a high dose of KCl 60 mmol/L and this was repeated at least 3 times until a sustained contraction was achieved in each ring. The cumulative concentration-dependent responses to phenylephrine (Phe) were expressed as a percentage of the contraction to KCl (60 mmol/L), which induces contraction through membrane depolarization. Endothelium-dependent and -independent vasodilations in response to acetylcholine (Ach; 10\textsuperscript{–9}–10\textsuperscript{–7} mol/L) and sodium nitroprusside (10\textsuperscript{–9}–10\textsuperscript{–4} mol/L) were expressed as a percentage of reduced contraction to Phe 10\textsuperscript{–6} mol/L. Contractile responses to Phe and endothelium-dependent vasodilatation in response to Ach were investigated also after pre-incubations with Tiron 1 mmol/L, polyethylene-glycol (PEG)-catalase 150 IU/ml and carboxy-2-phenyl-4,4,5,5-tetra-methyl-imidazoline-1-oxyl-3-oxide (Carb-PTIO) 0.1 mmol/L for 30 min before adding contractile agonists.

Measurement of Superoxide Production

Perivascular adipose tissue-derived superoxide formation was determined with minor modifications as previously described by Gao et al.\textsuperscript{9} Briefly, perivascular fat samples from lean and obese mice were equilibrated in Krebs solution (pH 7.4, 37°C) for 2 h. Thereafter they were pre-incubated in the presence and in the absence of apocynin (a NAPDH-oxidase inhibitor preventing p47phox subunit membrane assembly) 1 mmol/L, a superoxide scavenger Tiron 10\textsuperscript{–2} mol/L or scavenger of hydrogen peroxide, PEG-catalase 150 IU/ml. In order to stimulate superoxide anion production, perivascular fat samples were also pretreated with angiotensin II (Ang II) 10\textsuperscript{–6} mol/L for 30 min. The samples were then transferred into vials filled with Krebs enriched with luminol (250 μmol/L). The fat samples were dark adapted in 96-well plates at 37°C for 10 min. Superoxide was calculated from chemiluminescence measurements (Liquid Scintillation Counter, 1450 MicroBeta, Wallac, Boston, MA, USA). After the measurements, the samples were wet weighed with the results being reported as counts · mg\textsuperscript{–1} · min\textsuperscript{–1}.

Morphometry of Aorta and PVAT

Samples of abdominal aorta from lean and obese mice were fixed in 10% formalin and embedded in paraffin. Six-micron-thick cross-sections of the aortas were stained with hematoxylin and eosin. The morphology of aortic wall dimensions, the size of PVAT and the corresponding adipocytes were measured under light microscopy using Leica Qwin Standard software (Leica Microsystems Imaging Solutions, Cambridge, UK).
Total RNA was isolated from fat tissue using Trizol (Invitrogen, Carlsbad, CA, USA) and treated with DNAse 1 (deoxyribonuclease 1, Sigma Chemical, St Louis, MO, USA). RT of mRNA was done using ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). Quantitative RT-PCR was performed according to the manufacturer’s instructions with LightCycler carousel based system (Roche Diagnostics). Results were normalized with 18S.

The following primers were used: monocyte chemotactic peptide-1 (MCP-1) forward CGGAACCAAATGAGATCAG, reverse TCACAGTCCGAGTCAC; leptin forward AGACCAGATGCTTG; and 18S forward ACATCCAAGGCGAAG, reverse TTTTCGTCACTACCTCCCG.

Drugs
Acetylcholine, apocynin, Carb-PTIO, eosin, hematoxylin, PEG-catalase, Phe, sodium nitroprusside, Tiron and chemical components of the Krebs solution: NaCl, NaHCO\(_3\), glucose, KCl, CaCl\(_2\), MgSO\(_4\), KH\(_2\)PO\(_4\) were obtained from Sigma Chemical (USA). All concentrations used in organ chamber experiments are expressed as final molar (mol/L) concentration in the bath solutions.

Statistical Analysis
Data are presented as mean±SEM. Statistically significant difference in means were tested on 2-way ANOVA and on Student’s t-test. The difference was considered significant for P<0.05. The data were analyzed using GraphPad Prism, ver-
sion 4.02 (GraphPad Software, San Diego, CA, USA).

Results

Effect of Diet on Body Weight, Glucose Tolerance and Body Composition

Feeding of the high-fat diet enhanced body weight gain after 2 months as compared to a normal-fat diet (high fat vs normal fat, P<0.0001; Figure 1A). The significant increase in the body weight gain of the high-fat-fed mice was maintained until the end of the study. Blood glucose levels were markedly higher after 0 min, 15 min, 30 min, 60 min and 90 min in the high-fat-fed mice as well as the area under the curve (obese, 1,449 arbitrary units vs lean, 849 arbitrary units; P<0.001) in the oral glucose tolerance test as compared with normal-fat-fed mice after 8 months of treatment, indicating impaired glucose tolerance (Figure 1B). High-fat-fed mice had a marked increase in total body fat percentage measured on DEXA as compared to lean controls (Table).

Effect of Diet on Abdominal Aortic PVAT and Vascular Morphology

The area of PVAT measured on light microscopy was 3-fold increased in high-fat-fed mice as compared to lean controls (Figure 1; Table), and the adipocyte cross-sectional area was increased by 5-fold (Figure 1; Table). There was no difference between the groups in the lumen-to-wall ratio, a marker of vascular hypertrophy (Figure 1; Table).

Effects of Perivascular Adipose Tissue on Vascular Tone

Contractile responses to KCl were comparable between lean and obese mice both in the absence and in the presence of PVAT (lean(PVAT−), 0.44±0.06 g; obese(PVAT−), 0.45±0.05 g; lean(PVAT+), 0.51±0.06 g; obese(PVAT+), 0.41±0.06 g). Because the vasoconstrictions were comparable between the groups, the contractile response to Phe was normalized to vasoconstriction to KCl 60 mmol/L in each group. After precontraction induced by KCl, the concentration-dependent responses to endothelium-dependent vasodilator Ach were markedly enhanced in the presence of PVAT in lean mice (Figure 2A), whereas obese mice had significantly attenuated endothelium-mediated vasodilatation in the presence of PVAT (Figure 2B). After precontraction evoked by Phe, the concentration-dependent responses to endothelium-dependent vasodilator Ach were similar in the absence and in the presence of PVAT in lean mice (Figure 2C), while in the obese mice the presence of PVAT markedly impaired endothelium-mediated relaxation responses to Ach (Figure 2D). Responses to the endothelium-independent vasodilator sodium nitroprusside were similar in both groups, and PVAT did not influence the vascular responses to sodium nitroprusside (data not shown). Pre-incubation with a cell-permeable superoxide scavenger Tiron (Figure 3A) or PEG-catalase (Figure 3B) markedly enhanced the relaxation responses to Ach in obese mice, whereas pretreatment with Carb-PTIO did not alter dilator responses to Ach in obese aortas with perivascular fat (data not shown). Both pretreatments with
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Figure 3. Effects of perivascular fat on concentration response curves to acetylcholine in phenylephrine preconstricted obese aortas pretreated with (A) Tiron 1 mmol/L and (B) polyethylene-glycol (PEG)-catalase 150 IU/ml. Effects of perivascular fat on concentration response curves to acetylcholine in phenylephrine preconstricted obese aortas pretreated with (C) carboxy-2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (Carb-PTIO) 10^-4 mol/L or (D) PEG-catalase 150 IU/ml. Data given as mean ± SEM. PVAT, periadventitial adipose tissue.

Carb-PTIO (Figure 3C) or with PEG-catalase (Figure 3D) decreased relaxation responses to Ach in lean mice with PVAT. The contractile responses to Phe were markedly attenuated in the presence of PVAT in lean mice (Figure 4A), while its presence did not attenuate contractile responses to Phe in obese mice (Figure 4B). Pre-incubation with Tiron or with Carb-PTIO did not alter the PVAT-mediated response to Phe in lean mice (data not shown), whereas PEG-catalase pretreatment slightly but significantly reduced the anti-contractile properties of PVAT (Figure 4C). Obese mice with PVAT showed unaltered contractile responses to Phe (data not shown), whereas PEG-catalase pretreatment slightly but significantly reduced the anti-contractile properties of PVAT (Figure 4C). Pre-incubation with Tiron markedly attenuated the contractile responses to Phe (Figure 4D). Pretreatment with PEG-catalase markedly attenuated the contractile responses to Phe in aortas without PVAT taken from lean mice whereas pretreatment with Tiron or Carb-PTIO did not alter vasoconstriction to Phe (Figure 5A). In obese mice, pretreatment with PEG-catalase and with Tiron markedly reduced contractile responses to Phe in aortas without PVAT, whereas Carb-PTIO did not influence vasoconstriction to Phe (Figure 5B). In the aortas without PVAT taken from obese and lean mice, endothelium-dependent vasodilatation in response to Ach was not influenced either by pretreatments with Tiron or PEG-catalase but was markedly reduced by Carb-PTIO (Figures 5C, D).

ROS Production From Perivascular Adipose Tissue
Basal superoxide production was increased by 2-fold in obese mice as compared with lean mice (Figure 6A). Angiotensin II pre-incubation increased superoxide generation by 4-fold as compared with basal values in both mice groups (Figure 6A). Pre-incubation with a NADPH-oxidase inhibitor, apocynin, attenuated both basal and AngII-stimulated superoxide formation in lean mice and more pronouncedly in obese mice (Figure 6A). The basal hydrogen peroxide production from PVAT was significantly higher in obese mice as compared with lean mice (Figure 6B). Pre-incubation with AngII increased the levels of hydrogen peroxide by 10-fold in lean mice but only by 3-fold in their obese counterparts (Figure 6B).

Perivascular Adipocytokines and NADPH Oxidase mRNA
The MCP-1, leptin and Ncf2 (a gene encoding p67phox subunit of NADPH oxidase) mRNA was markedly increased in PVAT obtained from obese mice as compared with lean mice, whereas adiponectin mRNA was similar between both mice groups (Figures 6C–F).

Discussion
In the present study we investigated the contribution of oxidative stress derived from PVAT to vascular dysfunction.
Figure 4. Effects of perivascular fat on concentration response curves to phenylephrine in (A) lean and (B) obese mice. Effects of perivascular fat on concentration response curves to phenylephrine in (C) lean aortas pretreated with polyethylene glycol (PEG)-catalase 150IU/ml or (D) obese aortas pretreated with Tiron 1mmol/L. Data given as mean±SEM. PVAT, perivascular adipose tissue.

Figure 5. Contractile responses to phenylephrine in (A) lean or (B) obese aortas with periventricular adipose tissue (PVAT) removed and pretreated with Tiron, polyelectrolyte glycol (PEG)-catalase and carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxide (Carb-PTIO). Relaxation responses to acetylcholine in (C) lean or (D) obese aortas with PVAT removed and pretreated with Tiron, PEG-catalase and Carb-PTIO. Results expressed as mean±SEM.
Obesity increased ROS formation, expression of NADPH oxidase and pro-inflammatory adipocytokines in the PVAT. These changes were associated with decreased anti-contrac-
tile effect of PVAT and endothelial dysfunction. Thus, the present study suggests a pivotal role for PVAT in the pathogenesis of vascular dysfunction.

Interestingly, we showed that lean aortas responded to Ach differently depending on the presence of PVAT and the pre-
constrictor used. In concordance with a previous report,10 we here demonstrated that PVAT does not influence vasodilatory response in Phe-preconstricted aortas from lean mice. In contrast, when aortic rings were preconstricted with KCl, a greater vasodilatory response to Ach was observed in aortas with PVAT as compared with aortas without PVAT. The findings thus suggest that PVAT modulates endothelium-dependent and cholinergic-stimulated vasodilatation in response to depolarization. Elevated external K+ has been shown to depo-
larize vascular smooth muscle cell and activate Ca2+ influx via L-type Ca2+ channels, whereas α1-adrenoceptor stimulation with Phe has been shown to induce intracellular Ca2+ release from sarcoplasmic reticulum Ca2+ stores triggered by IP3 receptors.24 In a very recent study Van Hove et al demonstrated that aortic rings precontracted by Phe relax better and at lower nitric oxide concentrations than rings precontracted via L-type Ca2+ influx (high K+).25 Furthermore, the authors concluded that interventions influencing L-type Ca2+ influx during precontraction have an effect on the relaxing capacity of NO. Perivascular adipose tissue promotes vasodilatation and/or anti-contractility through tonic release of hydrogen sulfide,26 which has been shown to decrease Ca2+ influx through L-type Ca2+ channels in rat papillary muscles.27 Hence, the differences in vasodilatory response to Ach found in the present study can be explained, at least in part, by the different mode of action of the preconstrictors as well as the
capacity of perivascular tissue to secrete biologically active molecules that may influence Ca²⁺ metabolism in the vascular smooth muscle cell.

In the present study PEG-catalase impaired endothelium-dependent vasodilatation in the aortas of lean mice with PVAT, whereas in the aortas taken from obese mice PEG-catalase improved endothelium-dependent vasorelaxation. In contrast, PEG-catalase did not alter vascular relaxation in the aortic rings without PVAT. The findings thus suggest that PVAT-derivered hydrogen peroxide modulates vascular function and that this effect is dependent on obesity. We also observed greater production of hydrogen peroxide from PVAT taken from obese mice compared with lean controls. It has been shown previously that hydrogen peroxide released from PVAT exerts beneficial vascular effects in control animals.7 In good agreement, we noticed in the present study that pharmacological blockade of the vascular effects of hydrogen peroxide impaired Ach-induced vascular relaxation in the aortic rings with PVAT taken from lean mice. Several studies, however, have shown that hydrogen peroxide may also impair vascular function depending on its concentration, contractile status, vascular bed and animal model/species used.33 A very recent study by Greenstein et al showed in obese patients that the vascular functions of PVAT were improved after concomitant removal of hydrogen peroxide and superoxide.23 In the present study PEG-catalase improved endothelium-dependent vascular relaxation in aortic rings with PVAT taken from obese mice, suggesting that obesity promotes endothelial dysfunction via PVAT-derived hydrogen peroxide. In line with the present results, it has been shown previously that hydrogen peroxide evokes a greater vascular contraction in spontaneously hypertensive rats than in normotensive Wistar controls via mechanisms linked to increased thromboxane A₂ release, elevations in vascular smooth muscle Ca²⁺ levels and increased superoxide production.29 It has also been demonstrated that diet-induced obese C57Bl/6 mice show increased sensitivity to thromboxane A₂-mediated vasoconstriction.11 Therefore, it is tempting to speculate that the increased amount of hydrogen peroxide released from obese PVAT could promote endothelial dysfunction via increased vascular formation of contractile prostanoids and/or superoxide anions.

NADPH oxidase has been shown to be upregulated in the adipose tissue taken from obese mice while angiotensin II blockade reduces ROS formation, attenuates expression of NADPH oxidase subunits and ameliorates adipocytokine dysregulation.30 Therefore we pre-incubated a subgroup of isolated PVAT samples with angiotensin II in order to investigate the role of the renin–angiotensin system in the formation of ROS from PVAT. Both production of superoxide and hydrogen peroxide were stimulated by angiotensin II and markedly decreased by apocynin, a non-specific inhibitor of NADPH-oxidase. Additionally, we found that mRNA expression of Ncf2, a gene that encodes the p67phox subunit in the complex of NADPH oxidase, was markedly increased in the PVAT of obese mice.31 In line with the present findings, Gao et al showed recently that, in the PVAT, electric field stimulation leads to formation of superoxide anions and augmented vasoconstriction due to induction of NADPH oxidase.9

In the present study the gene expression of pro-inflammatory adipocytokines MCP-1 and leptin were markedly increased in the PVAT taken from obese mice as compared to lean controls. It has been shown previously that increased lipid accumulation and adipose cell enlargement are associated with increased expression of pro-inflammatory cytokine MCP-1, a key regulator of macrophage infiltration into the adipose tissue.32 Macrophages, in turn, abundantly secrete ROS via functional NADPH oxidases, thereby promoting both dysregulation of adipocytokines and endothelial cell damage.23 Hyperleptinemia is common in obesity and is independently associated with MCP-1 production, insulin resistance and cardiovascular diseases.34,35 Leptin has been shown to play an important role in the pathogenesis of obesity-induced endothelial dysfunction through promoting uncoupled eNOS.36 Therefore, the present findings strongly support the notion that a high-fat diet induces an inflammatory response in the perivascular adipose tissue, which, in turn, plays an important role in vascular remodeling and increased oxidative stress.37,38 In contrast to MCP-1 and leptin, adiponectin has been widely viewed as an anti-inflammatory and anti-oxidative adipocytokine that exerts favorable effects on the vascular wall.39 A short-term high-fat diet for 2 weeks markedly lowers the expression of adiponectin in C57Bl/6 mice, while serum adiponectin levels show a tendency to a paradoxical increase with age in New Zealand obese mice, a mouse model of metabolic syndrome.39,40 In the present study we did not detect any significant difference in adipocyte adiponectin mRNA expression between obese and lean mice, suggesting that, at least at the time point examined, adiponectin did not play a major role in the pathogenesis of endothelial dysfunction in diet-induced obese mice.

In conclusion the present findings suggest that PVAT promotes endothelial dysfunction in diet-induced obese C57Bl/6 mice via mechanisms that are linked to increased NADPH oxidase-derived oxidative stress and increased production of pro-inflammatory cytokines.

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