Adverse effects of the classic antioxidant uric acid in adipocytes: NADPH oxidase-mediated oxidative/nitrosative stress

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Sautin YY, Nakagawa T, Zharikov S, Johnson RJ. Adverse effects of the classic antioxidant uric acid in adipocytes: NADPH oxidase-mediated oxidative/nitrosative stress. Am J Physiol Cell Physiol 293: C584–C596, 2007. First published April 11, 2007; doi:10.1152/ajpcell.00600.2006.—Uric acid is considered a major antioxidant in human blood that may protect against aging and oxidative stress. Despite its proposed protective properties, elevated levels of uric acid are commonly associated with increased risk for cardiovascular disease and mortality. Furthermore, recent experimental studies suggest that uric acid may have a causal role in hypertension and metabolic syndrome. All these conditions are thought to be mediated by oxidative stress. In this study we demonstrate that differentiation of cultured mouse adipocytes is associated with increased production of reactive oxygen species (ROS) and uptake of uric acid. Soluble uric acid stimulated an increase in NADPH oxidase activity and ROS production in mature adipocytes but not in preadipocytes. The stimulation of NADPH oxidase-dependent ROS by uric acid resulted in activation of MAP kinases p38 and ERK1/2, a decrease in nitric oxide bioavailability, and an increase in protein nitrosylation and lipid oxidation. Collectively, our results suggest that hyperuricemia induces redox-dependent signaling and oxidative stress in adipocytes. Since oxidative stress in the adipose tissue has recently been recognized as a major cause of insulin resistance and cardiovascular disease, hyperuricemia-induced alterations in oxidative homeostasis in the adipose tissue might play an important role in these derangements.

redox signaling; nitric oxide; reactive oxygen species

URIC ACID IS AN INTERMEDIATE product of the purine degradation pathway in the cell. In most mammals, uric acid is degraded further by the enzyme uricase. In humans and Great Apes, the uricase gene was inactivated during hominoid evolution (44), and uric acid is the final metabolic product of purine catabolism. Humans are unique among mammalian species by having the highest basal blood level of uric acid ever seen (45) and the ability to develop hyperuricemia (22). Since the discovery in 1981 that urate is a powerful chemical antioxidant, which is present in human plasma in concentrations much higher than ascorbate (1), uric acid loses its antioxidant ability in the hydrophobic environment, urate can scavenge hydroxyl radical, singlet oxygen, and peroxynitrite, especially when combined with ascorbic acid or thiols (1, 2, 31). On the other hand, uric acid is not necessarily an antioxidant and, depending on the chemical milieu, may become a prooxidant. On one hand, in the extracellular environment, urate can scavenge hydroxyl radical, singlet oxygen, and peroxynitrite, especially when combined with ascorbic acid or thiols (1, 2, 31). On the other hand, uric acid loses its antioxidant ability in the hydrophobic environment (40). Moreover, it can form free radicals either alone (34) or in combination with peroxynitrite (51).

This study helps resolve the above paradox. We demonstrate that adipocyte differentiation is associated with increased uptake of uric acid and ROS accumulation and that elevated uric acid induced a further increase in intracellular ROS production in differentiated adipocytes, mediated by activation of NADPH oxidase (NOX). It is followed by redox-dependent stress signaling, a decrease in nitric oxide bioavailability, and oxidative modifications of proteins and lipids. Since oxidative stress in metabolic syndrome and obesity (8, 37, 45). Albeit there is tremendous complexity of these disorders, an unambiguous common pathogenetic feature for all of them is, paradoxically, an involvement of oxidative stress and oxidative modifications of proteins and lipids as well as redox-dependent low-grade inflammation (5, 13, 18, 53, 57).

Oxidative stress and inflammation in the adipose tissue induce an imbalance in the production of adipocyte-specific hormones and cytokines (adipokines) that contribute substantially to the development of insulin resistance and cardiovascular risk associated with obesity (6, 13, 57). Serum levels of uric acid are positively correlated with obesity (8, 45), especially visceral obesity (37). Although hyperuricemia is often considered as a secondary phenomenon in the metabolic syndrome (50), it has also been noticed to be an independent predictor of obesity and hyperinsulinemia (42, 58). Most importantly, it has been shown recently that uric acid has a causal role in the metabolic syndrome induced by fructose (41). The possibility that uric acid could have a direct effect on the adipose tissue was not considered and to the best of our knowledge remains unknown.

Physiological concentrations of soluble microcrystal-free uric acid induce gene expression of chemokines and growth factors, such as monocyte chemoattractant protein (MCP)-1 and PDGF (24, 56), and stimulate proliferation of vascular smooth muscle cells (VSMC) (25). The effects of urate may involve complex and poorly understood redox-dependent pathways. Urate-induced MCP-1 expression in VSMC was attenuated by antioxidants, suggesting involvement of redox-dependent mechanism (24). Available data suggest that uric acid is not necessarily an antioxidant and, depending on the chemical milieu, may become a prooxidant. On one hand, in the extracellular environment, urate can scavenge hydroxyl radical, singlet oxygen, and peroxynitrite, especially when combined with ascorbic acid or thiols (1, 2, 31). On the other hand, uric acid loses its antioxidant ability in the hydrophobic environment (40). Moreover, it can form free radicals either alone (34) or in combination with peroxynitrite (51).

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adipose tissue has emerged as a major cause of insulin resistance and imbalance in vascular homeostasis, hyperuricemia-induced alterations in oxidative homeostasis in adipose tissue might play a crucial role in these derangements.

**MATERIALS AND METHODS**

**Cell culture and treatments.** Preadipocyte 3T3-L1 cells obtained from ATCC (Manassas, VA) were maintained in high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and antibiotics. For differentiation, we treated confluent cells with 10 μg/ml insulin, 0.25 μM dexamethasone, and 0.5 mM IBMX for 2 days, followed by 3-day treatment with insulin alone. Aciduria solution for cell treatments was prepared in the prewarmed culture medium (1–15 mg/dl Ultrapure, a microcrystal-free endotoxin-free solution; Sigma, St. Louis, MO) and passed through a 20-μm sterile filter, as previously described (24).

**Detection of ROS and NO.** We assessed intracellular ROS using several independent methods. 1) We used the ROS-specific fluorescent probe 5(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Molecular Probes, Eugene, OR). At the end of treatments, cells were washed with Hank’s balanced salt solution (HBSS), followed by incubation in the presence of CM-H2DCFDA (5 μM) for 30 min in HBSS. The cells were then transferred to the original growth medium, and green fluorescence was measured using an Axiovert 200 inverted microscope (Carl Zeiss). For image acquisition and analysis of fluorescence intensity, we used the LD Achroplan ×40/0.60 Corr objective (Carl Zeiss), the AxioCam MRm charge-coupled device camera (CCD), an FITC filter (excitation 480/30 nm, emission 535/40 nm), and AxioVision (v.4.5) image acquisition and analysis software. All optical filters were obtained from Chroma Technologies. Images were acquired every 5 min at ambient temperature for at least 45 min, and fluorescence intensity was measured in cytoplasmic regions of 20–30 cells per field in 3–4 fields per experiment. Preliminary experiments showed that fluorescence stabilized after incubation for about 30 min in the presence of CM-H2DCFDA and remained stable for at least 15–20 min. Fluorescence intensities during this time interval were used for estimations of relative differences in ROS levels between groups. 2) We also measured superoxide generation using nitroblue tetrazolium (NBT) assay (46). Briefly, at the end of treatments, 0.2% NBT (Sigma) was added to the medium for 1 h, followed by washing of the cell monolayer with PBS and dissolving of water-insoluble reduced NBT (blue formazan) accumulated in cells in 50% acetic acid. The absorbance of blue formazan was measured at 560 nm using the Bio-Tek Powerwave 200 microplate reader (Bio-Tek Instruments, Winooski, VT). The contribution of the direct NBT reduction by uric acid in the culture medium to the total absorbance was negligible (~5% at the concentration 15 mg/dl when tested in the cell-free medium). 3) We also detected superoxide using the lucigenin-enhanced chemiluminescence method (see below). 4) We used Mn(II) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP), a superoxide scavenger and a cell-permeable mimetic of superoxide dismutase, to distinguish superoxide from another ROS.

The intracellular level of nitric oxide (NO) was measured using the NO-specific fluorescent probe 4-aminophenyl-2′,7′-dihalo-

**Immunofluorescence.** For immunofluorescence detection of the p40phox and p67phox translocation or specific urate transporter URAT1 expression, we cultured cells on coverslips. After treatments, cells were fixed in 3% (wt/vol) paraformaldehyde in PBS, quenched in 50 mM ammonium chloride and treated with 0.1% Triton X-100 for 10 min. p67phox and p40phox were stained with affinity-purified goat polyclonal NH2-terminal antibodies for p67phox (N-19) and p40phox (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) or URAT1 COOH-terminal polyclonal antibody (Alpha Diagnostics Interna-

**Measurement of NADPH oxidase.** NADPH oxidase activity was measured using the lucigenin-enhanced chemiluminescence method in crude cell homogenates and microsomal membrane fractions by following previously described procedures with minor modifications (15, 54). To prepare cell homogenates, the cell monolayer was washed three times with ice-cold PBS and scraped on ice in lysis buffer containing 20 mM K-phosphate buffer (pH 7.0), 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, and 5 μg/ml leupeptin, followed by homogenization with 100 strokes in a Dounce homogenizer on ice. For the isolation of microsomal membranes, cell homogenates were prepared in 250 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM PMSF, 10 μg/ml aprotinin, and 5 μg/ml leupeptin, followed by centrifugation at 1,000 g (10 min, 4°C). The pellet was discarded, and the supernatant was spun at 8,000 g (10 min, 4°C). The microsomal fraction was separated from cytosol by centrifugation of the supernatant at 105,000 g (45 min, 4°C). The pellet was resuspended in the homogenization buffer by using a Hamilton glass syringe. The cell homogenate and microsomal fraction were used immediately. The assay was started in an Orion microplate luminometer (Berthold Detection Systems) by automatic injection of the 150-μl reaction buffer [50 mM K-phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH] into 10 μl of the homogenate or membrane suspension (5–20 μg protein). Photon emission in response to superoxide generation was measured every 60 s with a 5-s signal integration time for 20 min. The activity is expressed in relative light units per milligram of protein. The protein concentration was measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

**Immunoblot detection of p67phox, p40phox, and phosphorylation of p38 and ERK1/2.** Phosphorylated and total p38 and ERK1/2 were detected by Western blotting with phosphorylation state-specific antibodies for p38 and ERK1/2 obtained from Cell Signaling Technology (Beverly, MA). p67phox and p40phox were detected with affinity-purified goat polyclonal antibodies from Santa Cruz Biotechnology. GAPDH was detected with monoclonal antibody were examined using a ×63 oil-immersion Plan-Apochromat ×63/1.4 objective under an Axioscop 2 imaging microscope (Carl Zeiss). Optical Z-sectioning and three-dimensional deconvolution (constrained iterative method) of the optical sections was performed using image acquisition and analysis software AxioVision (v.4.5).

**Detection of superoxide** from another ROS.
formed using NIH Image software. Ratios of phosphorylated kinases to total kinases or to housekeeping protein GAPDH were calculated.

**Protein nitrosylation.** To detect nitrosylated proteins, cell lysates were obtained as described above and proteins were resolved by SDS-PAGE, followed by electrophobting onto PVDF membrane. Nitrosylated proteins were detected by immunoblotting with monoclonal antibody to 3-nitrotyrosine (clone 39B6; Alexis Biochemicals, San Diego, CA). Densitometry of nitrosylated proteins was performed using NIH Image software.

**Ratiometric fluorescent analysis of lipid oxidation with oxidation-sensitive lipid peroxidation probe C11-BODIPY**

Lipid oxidation was measured using C11-BODIPY** (Molecular Probes), a validated lipid oxidation reporter molecule (9, 47). Differentiated adipocytes were incubated in HBSS in the presence of the probe (1 μM) for 30 min. The medium was then replaced with the HBBS-probe solution containing 7.5 mg/dl uric acid or vehicle. Time-lapse image capturing to the content of the oxidized lipids (9, 47). Differentiated adipocytes underwent oxidative stress, which is a well-established model of 3T3-L1 mouse adipocytes (16).

**RESULTS**

Increased ROS production and urate uptake are associated with the phenotype of differentiated adipocytes. We used a well-established model of 3T3-L1 mouse adipocytes (16). 3T3-L1 cells treated with the differentiation medium accumulated a remarkably high level of intracellular ROS, which is barely detectable in undifferentiated cells (Fig. 1A). As expected, adipocyte differentiation was associated with accumulation of lipids, increased expression of the adipogenic tran-
Hyperuricemia in vivo is induced by a variety of causes and medium affects the level of ROS produced by adipocytes. One of the major sources of ROS and oxidative stress in many cells and tissues is an activation of nonphagocyte-type NADPH oxidase. In response to various stimuli, this enzyme generates superoxide anions ($O_2^{-}$), which are converted subsequently into other ROS (4, 7, 19, 32). To test whether uric acid-induced ROS are superoxide...
dependent, we treated cells with uric acid in the presence of 25 μM MnTMPyP, a cell-permeable mimetic of superoxide dismutase. MnTMPyP abolished the effect of uric acid on ROS production (Fig. 2F), demonstrating the involvement of superoxide in the urate-induced ROS generation. In addition, the effect of uric acid was completely blocked by the general antioxidant N-acetylcysteine (NAC; 10 mM) as well as by apocynin (200 μM) and diphenylene iodonium (10 μM), structurally unrelated NOX inhibitors, whereas rotenone (100 μM), an inhibitor of the mitochondrial electron transport chain complex I, and thenoyltrifluoroacetone (100 μM), an inhibitor of the complex II, were without effect (Fig. 2G). These data suggest that the elevation in ROS abundance in adipocytes in response to uric acid depends on superoxide generation by NADPH oxidase but not by the mitochondrial respiratory chain, another major source of superoxide in the cell.

Next, we examined the effect of uric acid on the enzymatic activity of NADPH oxidase. With the use of lucigenin-enhanced chemiluminescent superoxide detection, we found that cellular homogenates of differentiated adipocytes are capable of NADPH-dependent O₂⁻ generation, which can be inhibited by apocynin or superoxide dismutase (Fig. 3A). These data indicate the presence of active NADPH oxidase in the adipocytes. Importantly, uric acid increased the superoxide dismutase-sensitive and apocynin-sensitive components of NADPH-dependent O₂⁻ production in a dose-dependent manner (Fig. 3B). Since active NADPH oxidase is a membrane-associated enzyme (4, 7, 32), we tested the effect of uric acid...
on the NADPH oxidase activity in microsomal membranes. Treatment of differentiated adipocytes with uric acid stimulated apocynin-sensitive NADPH-dependent O$_2^{-}$ generation in the microsomal fraction (Fig. 3C).

The mechanism for the activation of NADPH oxidase-dependent ROS production depends on the nature of the particular NOX isoforms and subunits involved in the formation of the active holoenzyme (4, 7, 32). It is known that 3T3-L1 adipocytes express NOX4 (33). What kind of cytoplasmic regulatory subunits are expressed in these cells remains unknown, although NOX4, in contrast to other NADPH oxidases, does not require cytoplasmic proteins for its activity (35). To define the isoforms of NADPH oxidase in 3T3-L1 adipocytes and better understand potential mechanism(s) of NOX activation by uric acid, we analyzed expression of mRNA for most known isoforms of the NOX family as well as cytoplasmic regulatory subunits involved in the formation of the classic phagocyte-type NADPH oxidase (gp91phox, p67phox, p47phox, p40phox, and p22phox).

The predominant mechanism of activation for many NOX isoforms is a stimulus-induced assembly of the active holoenzyme on the plasma membrane (7, 32, 52). Since we observed an expression of p47phox and p67phox, which are required for the activation of gp91phox and other NOX isoforms, and p40phox, which also may play a regulatory role, we assessed the effect of uric acid on translocation of p67phox and p40phox to the membranes. Immunoblot analysis of subcellular fractions of adipocytes treated with uric acid revealed that uric acid induced a dramatic dose-dependent increase in the content of p67phox in the microsomal fraction with a simultaneous decrease in the cytosol (Fig. 5A), indicating translocation of the subunit from the cytosol to membranes. An increase of p40phox content in the microsomal fraction was also visible (Fig. 5A).

Fig. 3. Uric acid induces NADPH oxidase (NOX) activation in adipocytes. A: apocynin- and superoxide dismutase (SOD)-sensitive superoxide generation. RLU, relative light units. B: urate-induced increase in NOX activity in homogenates. Data are expressed as the difference of the total activity of the homogenate and the activity in the presence of SOD (600 U/ml) or apocynin (200 μM). Values are means ± SE (n = 3). C: urate-induced NOX activation in microsomal membranes. Values are means ± SE (n = 3).
Fig. 4. Analysis of mRNA expression of NADPH oxidase isoforms and subunits in differentiated 3T3-L1 adipocytes. Total RNA was isolated from differentiated adipocytes, and, after treatment with DNase, 0.5 µg was amplified by RT-PCR with primers for all known mouse NOX isoforms and cytoplasmic subunits. Inverted images of agarose gels are shown; lane 1, adipocytes; lane 2, mouse kidney expressing many NADPH oxidases (positive control). Expected fragment size is shown above the gel image. A: NOX isoforms. B: cytoplasmic regulatory subunits with known isoforms. C: additional amplification of p22phox and p40phox. D: amplification of the GAPDH fragment (housekeeping gene) of the same RNA.

However, immunodetection of this subunit was difficult because of the low expression. Membrane translocation of p67phox and p40phox upon stimulation with uric acid was further confirmed by characterization of the trafficking using immunofluorescent detection, followed by microscopy with optical sectioning and three-dimensional deconvolution of the optical sections. Figure 5B shows the diffuse cytoplasmic localization of p67phox and p40phox in untreated cells and the rapid translocation of the immunoreactivity to intracellular vesicular structures (p67phox and p40phox) and to the plasma membrane (p40phox).

Urate-induced redox-dependent activation of ERK1/2 and p38 MAP kinases is mediated by NADPH oxidase. ROS are pivotal components of intracellular signaling pathways and regulate a variety of normal cellular functions and/or stress response mechanisms and antioxidant systems. To test whether urate-induced NOX-dependent ROS production is involved in activation of intracellular signaling pathways, we examined the effect of uric acid on the activation of two major pathways: p38 MAP kinase and ERK1/2 MAP kinase. Uric acid rapidly induced a dramatic but transient increase in the amount of activated (phosphorylated) but not total p38 and ERK1/2 (Fig. 6A). The activation was maximal at 5 min and decreased to basal levels after 30 min. Similar manipulations with uric acid-free medium did not produce any changes in p38 and ERK1/2 phosphorylation (not shown), demonstrating that the activation is not a result of medium change. To test involvement of NADPH-dependent ROS, we pretreated adipocytes with NAC, MnTMPyP, or apocynin before stimulation with uric acid. The inhibitors did not produce statistically significant changes in the phosphorylation of p38 and ERK1/2 MAPKs under basal conditions, but MnTMPyP and apocynin abolished or at least significantly attenuated the effect of uric acid (Fig. 6B). However, the effect of NAC was not statistically significant. Since NAC is a precursor of glutathione, it suggests that redox-dependent activation of p38 and ERK1/2 MAP kinases by uric acid may be relatively independent of the status of the glutathione system. It has been reported previously that redox-dependent activation of MAP kinases might be glutathione (and NAC)-dependent in some cases (17) and independent in others (55). Overall, our data demonstrate that urate-induced activation of ERK1/2 and p38 is at least partially redox dependent and is mediated by NADPH oxidase-induced superoxide.

NADPH-dependent decrease in NO bioavailability in adipocytes in response to uric acid. A decrease in NO bioavailability in endothelial cells appears to play an important role in the pathway by which uric acid causes hypertension, metabolic syndrome, and kidney disease in experimental models (30, 41). Endothelial (eNOS)- and inducible NO synthase (iNOS)-dependent NO is abundant in adipocytes; however, the biological role of NO in these cells is considered important but remains incompletely understood (12, 28). To address the possibility of redox-dependent intracellular effects of uric acid on the NO system in adipocytes, we measured NO production in differentiated adipocytes treated with uric acid. The level of NO in differentiated adipocytes was much higher than in undifferentiated adipocytes (Fig. 7A). An inhibitor of NO synthesis, Nω-nitro-L-arginine methyl ester (L-NAME), attenuated DAF-FM fluorescence, demonstrating NOS-dependent NO production in adipocytes (Fig. 7B). Long-term cell treatment with uric acid during adipocyte differentiation induced a dose-dependent decrease in NO bioavailability to levels detected in preadipocytes (Fig. 7C and Supplemental Fig. S2). Undifferentiated cells were not affected by treatment with uric acid (Fig. 7C). Uric acid added to differentiated adipocytes also induced a rapid decrease in NO levels (Fig. 7D and Supplemental Fig. S3). Probenecid prevented this decrease, suggesting that the effect of uric acid required its transport into the cytoplasm (Fig. 7D). The decrease in NO bioavailability was not due to changes in eNOS activation mediated by phosphorylation of Ser1177 (Supplemental Fig. S3), suggesting that the mechanism for
reducing NO level is not related to the common signaling pathway for eNOS activation. The decrease in NO bioavailability in response to uric acid was attenuated by NAC and apocynin, demonstrating that the effect of uric acid is mediated by oxidants and activation of NADPH oxidase (Fig. 7E).

Uric acid-induced oxidative modifications in adipocytes: protein nitrosylation and lipid oxidation. Given the high basal level of NO in differentiated adipocytes (Fig. 7A), the effect of uric acid to induce a simultaneous increase in ROS production (Fig. 2) and decrease in NO bioavailability (Fig. 7) suggested that urate-induced overproduction of ROS might result in formation of peroxynitrite as well as other reactive nitrogen species. Once formed, these radicals can induce oxidative modifications of proteins and lipids. To test this possibility, we performed immunoblot detection of nitrosylated proteins in cell lysates obtained from adipocytes treated with uric acid by using monoclonal antibody to 3-nitrotyrosine. Nitrination of tyrosine serves as a well-established marker or molecular “footprint” of reactive nitrogen species, including peroxynitrite (10). As shown in Fig. 8A, nitrosylated proteins are not detectable in untreated adipocytes, whereas long-term exposure to 1–15 mg/dl uric acid increased the amount of several nitrosylated proteins starting with the lowest tested concentration (Fig. 8A, bands 1–5) to detectable levels. Treatment of adipocytes with uric acid for 30 min increased nitrosylation of the protein(s) (Fig. 8B) with the apparent molecular mass similar to that of protein 2 detected after long-term stimulation with uric acid (Fig. 8A). Cell pretreatment with NAC, MnTMPyP, or apocynin prevented nitrosylation of this protein (Fig. 8B).

Thus superoxide generation and active NADPH oxidase are responsible for uric acid-induced protein nitrosylation in adipocytes.

To test formation of oxidized lipids, we used the oxidation-sensitive fluorescent fatty acid analog C11-BODIPY 581/591, which accumulates in lipid-containing structures of the cell and shifts fluorescence from red to green upon lipid peroxidation (9, 47). Green fluorescence of the probe as well as green/red fluorescence ratio gradually increased in untreated adipocytes, most likely because of high basal level of oxidants (Fig. 8, C and D). Uric acid induced a dramatic increase in green fluorescence of the probe almost immediately, and this increase continued for at least 45–60 min (Fig. 8, C and D, and Supplemental Fig. S4b) becoming visible after long-term treatment (Supplemental Fig. S4a). This effect was completely blocked by MnTMPyP or apocynin (Fig. 8, Cand D), demonstrating that lipid oxidation in response to uric acid was mediated by NOX-dependent superoxide.

DISCUSSION

The presented study is the first demonstration that uric acid exerts direct effects in adipocytes with potentially very important implications for our understanding of causal factors of the metabolic syndrome. It also provides a possible explanation for the paradox in which the chemical antioxidant urate is associated with diseases driven by oxidative stress. We have demonstrated that, at least in adipocytes, the redox-dependent effects of uric acid are mediated not by the redox chemistry of the urate compound but by the activation of intracellular oxidant production via NADPH oxidase.
So far, direct effects of soluble uric acid were characterized mostly in vascular smooth muscle cells and endothelial cells. In VSMC, uric acid activates critical proinflammatory pathways (23, 24) and stimulates cell proliferation (25, 49). In endothelial cells, uric acid decreases NO bioavailability (27, 30) and inhibits cell migration and proliferation, which are mediated in part by the expression of C-reactive protein (27). We observed urate-induced NADPH oxidase-dependent augmentation of ROS production and downstream ROS-mediated effects in differentiated adipocytes but not in preadipocytes. Adipocyte differentiation itself, which is accompanied by lipid accumulation and expression of differentiation markers in 3T3-L1 cells, induced basal ROS production, confirming recent findings (13). In addition, adipocyte differentiation enhanced the capacity of the adipocyte to uptake uric acid. Surprisingly, URAT1, previously considered as a kidney-specific urate transporter (39), is expressed in adipocytes and may be one of the transporters of uric acid in these cells. URAT1 is also expressed by the human vascular smooth muscle cell (48). The observation that ROS production in response to uric acid and downstream redox-dependent effects is sensitive to blockade of urate transport with probenecid and benzbromarone suggests that entry of uric acid into the cell is required for these effects.

The long-term and short-term effects of uric acid are similar. We observed dose-dependent effects of uric acid in our in vitro model of hyperuricemia in mouse adipocytes within a concentration range of 1–15 mg/dl, with distinct effects observed at concentrations as low as 1 mg/dl (60 μM). Mice, like most mammals, have active uricase eliminating most produced uric acid. Humans are not able to maintain the level of uric acid lower than about 3 mg/dl (20). Therefore, it is possible that the dose dependence of the effects of uric acid in human adipocytes will be different.

Uric acid induced activation of NADPH oxidase in crude homogenates and isolated microsomal membranes of adipocytes. Mechanisms of activation of NOX enzymes vary greatly depending on the spectrum of expressed isoforms and cytoplasmic regulators. NOX1–NOX3 require the presence of p22phox on the membrane and are more or less dependent on cytoplasmic subunits p47phox, p67phox, and p40phox, which translocate to the membrane and form with NOX and p22phox an active holoenzyme complex in response to cell stimulation (4, 32). NOX4 is a p22phox-dependent enzyme but does not require cytoplasmic subunits (35), and neither of these subunits is involved in the activation of NOX5 (4). Analysis of the mRNA expression for known isoforms of NADPH oxidase presented in this study revealed that differentiated adipocytes express NOX2, NOX3, and NOX4 as well as different types of cytosolic subunits, including p67phox and p47phox, which translocate to the membrane and form with NOX and p22phox an active holoenzyme complex in response to cell stimulation (4, 32). NOX4 is a p22phox-dependent enzyme but does not require cytosolic subunits (35), and neither of these subunits is involved in the activation of NOX5 (4). Analysis of the mRNA expression for known isoforms of NADPH oxidase presented in this study revealed that differentiated adipocytes express NOX2, NOX3, and NOX4 as well as different types of cytosolic subunits, including p67phox and p47phox, which translocate to the membrane and form with NOX and p22phox an active holoenzyme complex in response to cell stimulation (4, 32). NOX4 is a p22phox-dependent enzyme but does not require cytosolic subunits (35), and neither of these subunits is involved in the activation of NOX5 (4).
nant in fetal tissues (4, 32)], and the ubiquity of NOX4. These NOX isoforms can provide constitutive and stimulated ROS production in adipocytes, and different mechanisms may be involved in the activation of NADPH oxidase in response to stimulation. Our data on translocation of p67<sub>phox</sub> and p40<sub>phox</sub> in response to uric acid indicate the possibility of assembly of the classic phagocyte-type NADPH oxidase based on gp91<sub>phox</sub> or another NOX protein. Because we observed an increase in the content of p40<sub>phox</sub>, and because of the abundance of NOX4 in adipocytes, which is regulated predominantly at the level of transcription (4), the mechanisms underlying regulation of NOX expression are a promising topic for future experiments. The upstream signaling mechanism linking urate transport into the cell and formation of the active NOX complex in adipocytes remain largely unknown.

NOX-dependent superoxide generation in adipocytes induced in response to uric acid is associated with an increase in phosphorylation of p38 and ERK1/2, which can be blocked, at least partially, by scavengers of superoxide and inhibitors of NADPH oxidase. This indicates that redox-dependent signaling via cascades of MAP kinases mediates the effects of uric acid in adipocytes downstream from NADPH oxidase. Activation of p38 and ERK1/2 in response to uric acid also has been shown in VSMC (24, 56).

Fig. 7. Uric acid induces NOX-dependent decrease in NO bioavailability in adipocytes. A: nitric oxide (NO) induced fluorescence of the 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) probe. Images at top, phase contrast; images at bottom, fluorescence. B: suppression of NO production in adipocytes with N<sup>-</sup>nitro-L-arginine methyl ester (l-NAME). Cells were treated with l-NAME for 30 min before live imaging of NO production. Values are means ± SD (n = 3). *P < 0.05 (t-test). C: urate-induced decrease in NO bioavailability. 3T3-L1 cells were differentiated into adipocytes or incubated without differentiation factors in the presence or absence of varying concentrations of uric acid. Values are means ± SD (n = 3). The effect of uric acid is significant (P < 0.05, 1-way ANOVA). D: probenecid-sensitive acute effect of uric acid on NO bioavailability. Cells were pretreated with 1 mM probenecid for 30 min before addition of uric acid. Values are means ± SD (n = 3). The effect of uric acid is significant (P < 0.05, 1-way ANOVA). E: urate-induced decrease in NO bioavailability in adipocytes is prevented by antioxidants and inhibition of NOX. Cells were treated with 10 mM NAC or 200 μM apocynin for 30 min before addition of uric acid. Values are means ± SD (n = 3). The effect of uric acid is significant (P < 0.05, 1-way ANOVA). **P < 0.01 compared with untreated cells (U-test). &P < 0.05 compared with urate-treated group (U-test, n = 3).
Our results show that uric acid can increase oxidative stress in adipocytes such that local NO is inhibited and protein nitrosylation occurs. We detected an increase in the 3-nitrotyrosine content in several proteins in response to uric acid. The short-term effect of uric acid on the protein nitrosylation was blocked by antioxidants and inhibition of NADPH oxidase, demonstrating that NOX-dependent superoxide overproduction was responsible for protein nitrosylation. 3-Nitrotyrosine is a stable marker of reactive nitrogen species, including peroxynitrite, which forms in a rapid reaction between NO and superoxide with a near-diffusion-controlled rate (10). Peroxynitrite may be especially deleterious for adipocytes, because its diffusion rate in hydrophobic lipid environment is very high (29). Uric acid, indeed, induced lipid peroxidation, which was also prevented by NOX inhibition and superoxide scavenging. In addition, it is known from previous studies that

Fig. 8. Urate-induced NOX-dependent protein nitrosylation and lipid peroxidation in adipocytes. A: protein nitrosylation in 3T3-L1 cells treated with different concentrations of uric acid present during adipocyte differentiation. B: acute effect of uric acid on protein nitrosylation in differentiated adipocytes and its prevention by NAC, superoxide scavenging, and inhibition of NADPH oxidase. Differentiated adipocytes were treated with 15 mg/dl uric acid for varying periods of time or pretreated for 30 min with 10 mM NAC, 200 μM apocynin, or 25 μM MnTMPyP, followed by stimulation with uric acid (15 mg/dl) for 5 min. In A and B, graphs at bottom show the summarized data of densitometric measurements of the abundance of nitrosylated proteins. Values in A show the sum of all nitrosylated proteins for each dose of uric acid, normalized to the corresponding control. The effect of uric acid is significant (P < 0.05, 1-way ANOVA, n = 3). Values in B are the optical density of protein 2. The effect of uric acid is significant in both cases (P < 0.05, U-test, n = 3). The effects of all inhibitors compared with uric acid-treated cells are significant (P < 0.05, U-test, n = 3). C: detection of lipid peroxidation with C11-BODIPY581/591. Green fluorescence representing oxidized probe was detected every 60 s for 100 ms after addition of uric acid. D: ratiometric analysis of lipid oxidation. The ratio of green to red fluorescence measured every 60 s is shown.
in the hydrophobic environment, uric acid loses its antioxidant ability (40). In the presence of lipid peroxides, uric acid even becomes a strong prooxidant (3). Thus the intracellular environment of adipocytes, which is predominantly hydrophobic and has a high basal level of ROS, is largely unfavorable for manifestation of the antioxidant properties of uric acid, especially in the case of oxidants such as peroxynitrite. Moreover, uric acid induces intracellular production of superoxide via NOX, followed by formation of ROS and lipid peroxidation, which may further potentiate the chemical prooxidant ability of urate.

The adipose tissue is a source of low-grade inflammation in obesity, and this process plays a major role in the development of insulin resistance and vasculopathy leading to type II diabetes and increasing cardiovascular risk (6, 57). Obesity-associated oxidative stress in the adipose tissue has been recently recognized as a major causative factor for obesity-related inflammation and the metabolic syndrome (13). Uric acid blood levels positively correlate with obesity and body mass index (8, 37, 45) and predict the development of hyperinsulinemia, obesity, and type 2 diabetes (36, 43, 58). Therefore, based on the data presented in this study, hyperuricemia as a persistent condition associated with obesity may be a very important factor contributing to obesity-related oxidative stress.

Our results support previous epidemiological studies and animal models of hyperuricemia, which suggests an involvement of uric acid in the pathogenesis of the metabolic syndrome, and provide a possible molecular mechanism for this role based on the finding that soluble uric acid affects adipocytes directly by inducing NADPH oxidase-dependent oxidative stress. We suggest that hyperuricemia can be one of the causal factors inducing oxidative stress followed by a proinflammatory process and endothocyte dysfunction in the adipose tissue, thereby contributing to the pathogenesis of the metabolic syndrome and cardiovascular disease.

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