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

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Submitted 4 December 2006; accepted in final form 5 April 2007

Sautin YY, Nakawawa 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 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), it is widely accepted that high blood levels of uric acid in humans carry an evolutionary advantage and that uric acid is a major antioxidant that protects cardiac, vascular, and neural cells from oxidative injury (14, 53).

On the other hand, hyperuricemia even without crystal deposition and gout is strongly associated with cardiovascular disease, kidney disease, and hypertension, increasing the risk of mortality (21). Hyperuricemia is also common in the 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).

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...
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. Uric acid solution for cell treatments was prepared in the prewarmed cell 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 (DCFDA; Molecular Probes, Eugene, OR). At the end of treatments, cells were washed with Hanks’ 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 AxiosCam 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 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 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. 3) We also detected superoxide using the lucigenin-enhanced chemiluminescence method (see below). 4) We used Mn(II) tetakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP), a superoxide scavenger and a cell-permeable mimic 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-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate (DAF-FM diacetate; Molecular Probes, Eugene, OR). At the end of treatments, cells were washed with Hanks’ 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 AxiosCam 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. 3) We also detected superoxide using the lucigenin-enhanced chemiluminescence method (see below). 4) We used Mn(II) tetakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP), a superoxide scavenger and a cell-permeable mimic of superoxide dismutase, to distinguish superoxide from another ROS.

**Immunofluorescent detection of p40phox and p67phox.** We used the ROS-specific fluorescent probe 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate (DAF-FM diacetate; Molecular Probes, Eugene, OR). At the end of treatments, cells were washed with Hanks’ 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 AxiosCam 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. 3) We also detected superoxide using the lucigenin-enhanced chemiluminescence method (see below). 4) We used Mn(II) tetakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP), a superoxide scavenger and a cell-permeable mimic of superoxide dismutase, to distinguish superoxide from another ROS.

**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 EDTA, 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 EDTA, 150 mM sucrose, 5 mM lucigenin, and 100 μM NADPH) into 10 μl of the homogenate or membrane suspension (5–20 μg protein). Protein 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).

**Western blot analysis.** Protein samples of cell lysate were mixed (1:1) with Laemmli sample buffer and boiled for 5 min. Protein samples were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 containing 5% nonfat dry milk, followed by incubation with primary antibody. Membranes were washed three times and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The immunocomplexes were visualized by chemiluminescence with the Phototope Western blot detection system (Cell Signaling Technology). The images were digitized using the AlphaEase FluorChem digital imaging system (Alpha Innotech, San Leandro, CA). Band densitometry was performed using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD). The protein content of the supernatants was measured, and 20-μg protein samples of cell lysate were mixed (1:1) with Laemmli sample buffer and incubated at 95°C for 5 min. Proteins were resolved by SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 containing 5% nonfat dry milk, followed by incubation with primary antibody. Membranes were washed three times and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The immunocomplexes were visualized by chemiluminescence with the Phototope Western blot detection system (Cell Signaling Technology). The images were digitized using the AlphaEase FluorChem digital imaging system (Alpha Innotech, San Leandro, CA). Band densitometry was performed using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD).
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 electoblotting 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**390/490** (Molecular Probes), a validated lipid oxidation reporter molecule (9, 47). The probe accumulates readily in membrane and lipids, and in the presence of oxidized lipids its fluorescence shifts from red to green proportionally to the content of the oxidized lipids (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 HBSS-probe solution containing 7.5 mg/dl uric acid or vehicle. Time-lapse image capturing was started immediately following addition of uric acid at excitation/emission of 490/510 nm (the oxidized form, green emission of 580/600 nm (the nonoxidized probe, red fluorescence) and was performed at ambient temperature using the ratio/FRET (fluorescence resonance at excitation/emission of 490/510 nm (the oxidized form, green fluorescence).

For image acquisition, we used the Cell A-Plan ×20 objective (Carl Zeiss) and the AxioCam MRm CCD camera. Time-lapse ratiometric image acquisition was performed every 60 s at ambient temperature using the ratio/FRET (fluorescence resonance energy transfer) module of SlideBook 4.1 software (Intelligent Imaging Innovations, Denver, CO). Exposure time for both green and red fluorescence was 100 ms in all experiments. Fluorescence intensity was measured in at least 12 regions of interest for each recording.

**Real-time quantitative RT-PCR.** Total RNA for quantification of mRNA expression for adiponectin and peroxisome proliferator-activated receptor (PPAR)-γ was extracted using Trizol reagent (Invitrogen). Trace DNA was removed using a DNA-free kit (Ambion, Austin, TX). Total RNA (1 μg) was converted to cDNA, and quantitative real-time RT-PCR was performed using the SuperScript III Platinum Two-Step qRT-PCR kit with SYBR green (Invitrogen) with primers optimized for real-time PCR: mouse PPAR-γ (designed based on GenBank accession no. NM_011146), 5′-GGAAAGACAACGAGGAGAAGTTCCAAGCTCATTTGATGCTCCAC-3′ (forward) and 5′-AACTGGCCACCTTGAAAACT-3′ (reverse); mouse adiponectin (clone MADI 1147; Alexis Biochemicals).

**RT-PCR.** URA1 and subunits of the NADPH oxidase isoforms were detected by RT-PCR using SuperScript One-Step RT-PCR kit with Platinum Taq (Invitrogen). URA1 was detected with a pair of primers amplifying a 170-bp fragment of the mouse URA1 (GenBank NM_009203): 5′-ACAGAGGAGTGTCGTGCTCAAC-3′ (forward) and 5′-ACAGACATGGAGATGCTGGTC-3′ (reverse). GAPDH was used as a housekeeping gene. A 104-bp fragment of the mouse GAPDH was amplified simultaneously using the primers 5′-GGTTGAGAAGACCAAGAAATA-3′ (forward) and 5′-AACCTGGCCACCTTGTGAAAAT-3′ (reverse); mouse adiponectin (clone MADI 1147; Alexis Biochemicals).

**URATE-INDUCED OXIDATIVE STRESS IN ADIPOCYTES**

**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 adiogenic tran
Total urate concentration in the medium was 200 μM (Fig. 1A). ROS were detected using live imaging with the fluorescent probe 5(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate-acetyl ester (H2DCFDA). ROS-induced fluorescence is shown at top; merged images of the fluorescence and differential interference contrast (DIC) are shown at bottom. B: uptake of [14C]urate by 3T3-L1 cells (preadipocytes and differentiated adipocytes). Cells were incubated with the mixture of the labeled and nonradioactive uric acid (50–400 μM), and incorporated radioactivity was measured by scintillation counting. Values are means ± SD (n = 4). *P < 0.05 (U-test). C: effect of probenecid on uptake of [14C]urate by 3T3-L1 cells (time course). Total urate concentration in the medium was 200 μM. Values are means ± SD (n = 4). *P < 0.05 (U-test). D: mRNA expression for URAT1 and GAPDH in adipocytes. Total RNA from differentiated adipocytes and kidneys of C57Bl6 mice (positive control) was analyzed by RT-PCR. Inverted images of agarose gels are shown. NTC, no-template control. E: immunofluorescent localization of URAT1 in differentiated adipocytes. Merged images of green (URAT1 immunoreactivity) and blue (4,6-diamidino-2-phenylindole, DAPI) fluorescence are shown at top. DIC images are shown at bottom. Membrane and vesicular localization of URAT1 is indicated by arrows and arrowheads, respectively.

Fig. 1. Phenotype of differentiated adipocytes is associated with increased reactive oxygen species (ROS) production and uptake of uric acid (UA). A: ROS production by differentiated adipocytes (DIF) and preadipocytes (undifferentiated, UD). ROS were detected using live imaging with the fluorescent probe 5(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate-acetyl ester (H2DCFDA). ROS-induced fluorescence is shown at top; merged images of the fluorescence and differential interference contrast (DIC) are shown at bottom. B: uptake of [14C]urate by 3T3-L1 cells (preadipocytes and differentiated adipocytes). Cells were incubated with the mixture of the labeled and nonradioactive uric acid (50–400 μM), and incorporated radioactivity was measured by scintillation counting. Values are means ± SD (n = 4). *P < 0.05 (U-test). C: effect of probenecid on uptake of [14C]urate by 3T3-L1 cells (time course). Total urate concentration in the medium was 200 μM. Values are means ± SD (n = 4). *P < 0.05 (U-test). D: mRNA expression for URAT1 and GAPDH in adipocytes. Total RNA from differentiated adipocytes and kidneys of C57Bl6 mice (positive control) was analyzed by RT-PCR. Inverted images of agarose gels are shown. NTC, no-template control. E: immunofluorescent localization of URAT1 in differentiated adipocytes. Merged images of green (URAT1 immunoreactivity) and blue (4,6-diamidino-2-phenylindole, DAPI) fluorescence are shown at top. DIC images are shown at bottom. Membrane and vesicular localization of URAT1 is indicated by arrows and arrowheads, respectively.

Urate-induced production of ROS in adipocytes. Next, we determined whether the presence of soluble uric acid in the medium affects the level of ROS produced by adipocytes. Hyperuricemia in vivo is induced by a variety of causes and can be chronic or acute (26). To test long-term effects of uric acid, we incubated cells with varying concentrations of uric acid during adipocyte differentiation. To detect acute effects, uric acid was added to differentiated adipocytes for short periods of time (5–30 min). The presence of 1–15 mg/dl uric acid during the differentiation of adipocytes produced a moderate but significant increase in ROS production as determined using the NBT assay, whereas no effect of uric acid was detected in untreated undifferentiated cells or in incompletely differentiated cells (Fig. 2A). Similar results were obtained when ROS were detected using the fluorescent probe H2DCFDA (Fig. 2B and C). When differentiated adipocytes were treated with varying concentrations of uric acid for 30 min, the effect on ROS production was even more pronounced than after long-term stimulation (Fig. 2D). To determine whether transport of uric acid into adipocytes is required for stimulation of ROS production, we pretreated cells with probenecid and benz bromarone, two structurally unrelated OAT inhibitors of the transmembrane transport of urate (39). As shown in Fig. 2E, both inhibitors prevented stimulation of ROS generation in adipocytes in response to uric acid, suggesting that uric acid must enter the cell to induce ROS production.

Involvement of NADPH oxidase in urate-induced ROS production in 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 variety of stimuli, this enzyme generates superoxide anions (O2•−), 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 its formation (Fig. 4). Differentiated 3T3-L1 adipocytes predominantly express NOX4 (Fig. 4A), which is in agreement with the previously published observation (33). However, in addition to NOX4, we were able to detect NOX3 and NOX2 (gp91\(_{phox}\)) but not NOX1 (Fig. 4A), DUOX1, and DUOX2 (not shown). Among other proteins involved in the formation of active NADPH oxidase, we detected quite high expression of mRNAs for NOXA2 (p67\(_{phox}\)), NOXO1, and NOXO2 (p47\(_{phox}\)) (Fig. 4B). Expression of mRNA for p40\(_{phox}\) was lower than for other proteins, and this mRNA could be reliably detected only after increasing the number of amplification cycles (Fig. 4C). Surprisingly, p22\(_{phox}\), which is an important activator for most NOX enzymes, was also detected only after additional amplification (Fig. 4, B and C), and it was expressed at a level comparable with p40\(_{phox}\). Thus 3T3-L1 adipocytes express several isoforms of NADPH oxidase: NOX4, NOX3, and NOX2 with several regulatory subunits, including those involved in the formation of the classic phagocyte-type NADPH oxidase (gp91\(_{phox}\), p67\(_{phox}\), p47\(_{phox}\), p40\(_{phox}\), and p22\(_{phox}\)).

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 p47\(_{phox}\) and p67\(_{phox}\), which are required for the activation of gp91\(_{phox}\) and other NOX isoforms, and p40\(_{phox}\), which also may play a regulatory role, we assessed the effect of uric acid on translocation of p67\(_{phox}\) and p40\(_{phox}\) 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 p67\(_{phox}\) 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 p40\(_{phox}\) content in the microsomal fraction was also visible (Fig. 5A).
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 kinases. Uric acid rapidly induced a dramatic but transient increase in the amount of activated (phosphorylated) but not total p38 and ERK1/2 MAP kinases. 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 kinases. 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 NADPH oxidase-generated ROS are not redox 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-generated 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 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 at 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. Nitration 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.

Fig. 5. Translocation of p67phox and p40phox in adipocytes in response to uric acid. Adipocytes were cultured and differentiated in 10-cm dishes (for cell fractionation) or on coverslips (for immunofluorescence). At the end of incubation with 5–15 mg/dl uric acid, cells were processed as described in MATERIALS AND METHODS. A: urate-induced changes in the content of p67phox and p40phox in microsomal fractions, cytosol, and total lysate. Densitometry values are changes in the optical density in response to uric acid, normalized to the corresponding control. *P < 0.05 compared with control (U-test, n = 3). WB, Western blot. B: effect of uric acid on p67phox and p40phox localization in adipocytes. Shown are 1-μm optical sections (X, Y). Images 1 and 2, immunofluorescence of p67phox; images 3 and 4, merged images of DIC and DAPI staining to show nuclei.
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 benzbrromarone 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 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 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 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 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 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 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 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 cytop
dant 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 p67phox and p40phox in response to uric acid indicate the possibility of assembly of the classic phagocyte-type NADPH oxidase based on gp91phox or another NOX protein. Because we observed an increase in the content of p40phox, 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-aminofluorescein diacetate (DAF-FM) probe. Images at top, phase contrast; images at bottom, fluorescence. B: suppression of NO production in adipocytes with Nω-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 < 0.01 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...
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 endothelial dysfunction in the adipose tissue, thereby contributing to the pathogenesis of the metabolic syndrome and cardiovascular disease.

GRANTS
This work was supported by National Institutes of Health Grants DK-52121 and HL-68607 (to R. J. Johnson), the Gatorade Research Fund (to Y. Y. Sautin), and an American Lung Association of Florida Grant (to S. Zharikov).

REFERENCES