Supplemental Materials and Methods, Legend to Figures, Table I, and Legend to Figures on line only.

Materials and Methods

Chemicals

Phorbol 12-myristate 13-acetate (PMA), 2’, 7’-dichlorofluorescin diacetate (DCFH), amiloride, vitamin E, apocynin and diphenyleneiodonium (DPI) were from Sigma (St. Louis, MO, USA). Human 2-chain high molecular weight uPA activity standard, ATF (amino terminal fragment)-uPA and fluorescein conjugated high molecular weight human urokinase were from American Diagnostica Inc. Active 2-chain high molecular weight mouse uPA, recombinantly produced in insect cells, was from Innovative Research, MI, USA. Mouse anti-human CD87 (clone VIM5) and mouse IgG1 were from BD Biosciences, Pharmingen. Plasmin, plasminogen and S-2251 chromogenic substrate for plasmin were from Chromogenix, Milano, Italy. PBS, DMEM, RPMI-1640 medium, FCS (heat-inactivated at 56 °C for 30 min), penicillin, streptomycin, nystatin, L-glutamine, and sodium pyruvate were from Biological Industries (Beth Haemek, Israel).

Cells

Exponentially growing human myeloid leukemia cell line THP-1 at a starting density of 2x10^5/mL was cultured for 3-4 days in RPMI-1640-glutamine medium supplemented with 5% FCS, 100,000 U/L penicillin, 100µg/mL pyruvate, 100 mg/L streptomycin and 10 ng/mL of PMA, at 37°C in a
humidified incubator (5% CO2, 95% air). Cell viability measured by trypan blue was >90%. The cells were incubated with serum-free medium containing bovine serum albumin 0.2% and increasing concentrations of human uPA in the range of 5-40 nmol/L for 24 hours at 37°C, followed by analyses of cellular oxidative stress and PON2.

**Mice Studies**

Balb/C mice, C57Bl/6 mice, p47phox−/− mice, (15th generation on the C57Bl/6 background, a generous gift from Dr. Stephen M. Holland, Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA), uPAR−/− mice on the C57Bl/6 background (originally purchased from Jackson Laboratories, were a generous gift from Dr. Abd A. Higazi from the Department of Clinical Biochemistry at Hebrew University-Hadassah Medical School, Jerusalem, Israel), and PON2−/− (5th generation on the C57Bl/6 background, a generous gift from Dr. Srinivasa T. Reddy, Atherosclerosis Research Unit, Division of Cardiology, Department of Medicine at UCLA, Los Angeles, CA, USA) were sacrificed and peritoneal macrophages were harvested as described below. The experimental protocol was approved by the Animal Care and Use Committee of the Technion, No. IL-005-01-2005.

**Mouse peritoneal macrophages (MPM)**

MPM were harvested from mice 3 days after intraperitoneal injection of 3 ml thioglycollate 4%. The cells were washed (x2) with PBS, re-suspended at a
concentration of $1 \times 10^6$ cells/ml in DMEM containing 10% FCS, 100,000 U/L penicillin, 100 mg/L streptomycin, 2 mmol/L glutamine, and 100 µg/ml pyruvate. The cell suspension was dispensed into plastic Petri dishes and incubated in a humidified incubator (5% CO2, 95% air) at 37°C for 2 hours. The dishes were washed once with 5 ml DMEM to remove nonadherent cells, and the monolayer was then incubated under similar conditions prior to the beginning of the experiment. Cells were pooled from each individual mouse and were analyzed separately. MPM were then incubated with 20 nmol/L of mouse active uPA. Cell viability by trypan blue was >90%.

**Plasmin generation**

An amidolytic assay was performed to measure the activity of uPA and plasminogen activation, using the chromogenic substrate S-2251. Increasing concentrations of uPA were incubated for 20 minutes at 37°C with plasminogen (15µg/mL). Then, 100µg/mL of the plasmin substrate S-2251 was added and the optical absorbance obtained at 405 nm was recorded immediately and after 3 minutes of incubation. Generated plasmin was quantified using a calibration curve performed with purified plasmin (**Figure I**, please see http://atvb.ahajournals.org). uPA induced a dose-dependent plasminogen activation and plasmin generation, which reached saturation at 40 nmol/L of uPA (**Figure II**, please see http://atvb.ahajournals.org). Addition of 2.5 µmol/L of amiloride prevented plasmin generation. Similarly,
incubation of mouse uPA (20nmol/L) with plasminogen (15\(\mu\)g/mL) resulted in generation of 9.12 ± 0.5 of plasmin, as revealed after the addition of S-2251. These data evidence that both human and mouse uPA used in the present study are active. On the contrary, no plasmin was generated upon incubation of plasminogen with ATF-uPA, evidencing thus that it is not contaminated with uPA or with plasmin-like activity. Addition of S-2251 to human uPA, mouse uPA, or ATF, without the addition of plasminogen, revealed that these compounds were pure as they lack S-2251 activity. Incubation of S-2251 with uPA without the addition of plasminogen, for up to 60 minutes revealed a negligible increase in plasmin generation (from 0 to 0.02, 0.026, and 0.041 OD at 405nm, after incubation of 0, 3, 30, and 60 minutes, respectively). No such an increase could be found upon using mouse uPA.

Plasmin generation by cells grown under different experimental conditions was determined in conditioned media (CM) that was incubated with 100 \(\mu\)g/mL S-2251 at 37°C for 30 minutes, after which the OD at 405 was measured.

**Detection of intracellular oxidative stress**

Intracellular oxidative stress was assayed through the oxidation of 2’, 7’-dichlorofluorescin diacetate (DCFH-DA, Sigma) using FACS (FACS-SCAN, Becton Dickinson, San Jose, CA, USA), as previously described \(^1\).
Lipid peroxides

Formation of lipid peroxides was assayed using the CHOD iodide method 2.

Superoxide anion production

Production of superoxide anion was measured by the cytochrom C reduction assay, as previously described 3.

Cell Mediated LDL Oxidation

LDL was separated from plasma of normal healthy volunteers by discontinuous density-gradient ultracentrifugation. Macrophages were incubated with LDL (100 µg of protein/mL) in the presence of 5 µmol/L CuSO₄ for 5.5 h at 37°C. Cell-mediated LDL oxidation was determined by the TBARS assay 4.

Reverse transcriptase polymerase chain reaction (RT-PCR) for mouse PON2 and PON3, and for human and mouse p47phox

Total RNA extraction and RT-PCR reaction was carried out as previously described 21. Products of the RT reaction were subjected to PCR amplification. DNA sequences of upstream (UP) and downstream (DP) primers and PCR protocols are summarized in the table. β-Actin cDNA product was used as a standard to equivalent levels of total RNA subjected to RT-PCR for the other genes.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>PCR Primer Sequences</th>
<th>PCR Protocol</th>
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<tr>
<td>Mouse PON2</td>
<td>UP: 5-CACTGCTTTATCTTTATGTCGTG-3</td>
<td>94 °C/15 s</td>
</tr>
<tr>
<td></td>
<td>DP: 5-GAAGCAGACAGAGCCGTTGTC-3</td>
<td>57°C/30 s</td>
</tr>
<tr>
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<td>70°C/15 s</td>
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UP indicates upstream primer; DP, downstream primer.

**Reverse transcriptase quantitative polymerase chain reaction (Q-PCR) for human PON2**

Total RNA was extracted with Epicentre commercial kit (Tamar, Israel). cDNA was generated from 1 μg of total RNA using Thermo Scientific commercial kit (Tamar, Israel). Products of the RT were subjected to Quantitative PCR using TaqMan Gene Expression Assays. Quantitative PCR was performed on the Rotor-Gene 6000 system (Corbett Life science, Australia). The TaqMan sequences for PON2 gene expression were: Sense primer: CGACTTAAAGCCTCCAGAGAA; Anti-sense primer: GGGAAATTTTAGACCCACACTAAA; Probe: TAGACCTTCCACACTGCCCACCTGA. To normalize the data obtained for PON2 expression, the amount of β-actin mRNA was measured by quantitative
PCR as internal standard in all treatments. The primers and probes for human PON2 and β-actin were designed by "PrimerDesign" (United Kingdom).

**Paraoxonase 2-associated (lactonase) activity**

Cells (5×10^6) were washed and suspended into 0.5ml Tris buffer (25 mM Tris/HCL, pH 7.6, 1 mM CaCl₂), sonicated for 40 seconds at 80w on ice, and the protein concentration was determined. Enzyme lactonase activity was measured using 200 µl of sonicate (200 to 300 µg protein) per ml assay mixture, using dihydrocumarin (DHC) as substrate. The absorbance was monitored at 0 and 5 minutes after substrates addition. Measurements were as follows: 1 U of lactonase activity= 1 µmol of DHC hydrolyzed/minute.

**Paraoxonase 3-associated statinase activity**

Cell sonicate was prepared as described above. Lactonase activity using lovastatin was determined by high-performance liquid chromatography after cell sonicates incubation with lovastatin for 4 hours. Measurements were as follows: 1 U of PON activity= 1 pmol of lovastatin hydrolyzed/minute.

**Cell lysate preparation**

Subconfluent THP-1 macrophages were stimulated with uPA under the specific experimental conditions. Then, cells were washed, scraped in 2 ml of PBS, and pelleted (1000g for 10 minutes at 4°C). Pellets were suspended in 0.2-0.5 mL of lysis buffer [50mmol/L Tris Base pH 7.5, 150mmol/L NaCl, 5mmol/L EDTA, 0.5% NP-40, protease cocktail inhibitor (Sigma)].

**Immunoblotting**
Cell lysates were separated electrophoretically by SDS-PAGE (7.5% acrylamide and 10% acrylamide, respectively) and transferred to nitrocellulose membranes. Membranes were incubated with the following primary polyclonal antibodies: anti-PON2 (Research Genetics, Inc, Huntsville, Ala), and anti-β-Actin (Sigma). Horseradish peroxidase-conjugated with goat-anti-rabbit (Chemicon international) or gout-anti-mouse (Jackson Immunoresearch) were used as secondary antibodies. Bound antibodies were detected using enhanced chemiluminescence reagent (Beth Haemek, Israel) according to the manufacturers' instructions, and visualized by autoradiography. The signal intensity of the bands was quantified by densitometric scanning.

**Confocal microscopy analysis**

Cells were grown on 1.5mm cover-slide. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Permeabilized cells were stained directly with rabbit anti-mouse PON2 followed by goat anti-rabbit antibody conjugated to cy-3 (Jackson Immuno Research) and visualized in red. Topro (Invitrogen), visualized in blue, was used for counterstaining the macrophage nuclei. Negative controls consisted of slides treated with the second antibody alone, or with pre-immune serum. Slides were examined using radiance 2100 – confocal imaging system (Bio-Rad) under a x60 objective, and visualized by a Green HeNe (543nm) laser and red laser diode (637nm). Confocal images were converted to a Tiff format.
Statistical Analyses

Each separate experiment was performed in triplicate, and each individual experiment was replicated 3 times (n=3) in order to achieve statistical meaning. Statistical analyses used Student’s t test for comparing differences between the 2 groups, and one-way ANOVA followed by the Student-Newman-Keuls test was used for comparing differences between multiple groups.

Legend to Figures

Figure 1: The effect of uPA on macrophage PON2 expression. THP-1 cells were incubated for 24 hours without (control) or with increasing concentrations of human uPA (10-40nmol/L). (A) PON2 protein expression as determined by Western blot. The corresponding blots were stripped and developed with β-actin monoclonal antibody for assessment of equal loading. The figure shows a representative blot and the mean± SD of integrated densitometric data from 3 separate experiments. (B, C, D, E) Confocal laser scanning microscopic staining view of PON2 protein in THP-1 macrophages, which were incubated for 24 hours without and with uPA 20nmol/L. Projection of 12 z section with steps of 0.820 µm for nuclear stain (blue), PON2 (Red), and Merge. Incubation with PON2 pre-immune serum was used as control. All images were taken under the same conditions (Frame Rate 0.11 Hz; Line Frequency 166.0 Hz). (F) PON2 lactonase activity determined by
spectrometry at O.D 270nm for hydrolysis of dihydrocumarin as a substrate. Results are expressed as mean ± SD (n=3 separate experiments), *p<0.01 vs. control (without uPA).

(G) PON2 mRNA expression in macrophages was determined by quantitative real time PCR amplification as described in "Methods". The housekeeping gene β-actin was used as a control. Results are expressed as mean ± SD (n=3 separate experiments), *p<0.01 vs. control (without uPA).

**Figure 2:** Requirements for uPA proteolytic activity and binding to uPA-R (CD87) in the uPA-induced increase in macrophage PON2 expression. THP-1 cells were incubated without (Control), with human uPA (20nmol/L), with ATF-uPA (20nmol/L), or with uPA in the presence of amiloride (2.5µmol/L), anti CD87 purified mouse anti human antibodies, or mouse IgG, for 24 hours. Cellular PON2-associated lactonase activity (A) and protein expression (B) were determined as described in the “Methods” section. Results represent mean ± SD of three experiments. *p<0.01 vs. control (without uPA).

MPM harvested from six C57Bl/6 or six uPAR−/− mice were incubated without (Control) or with mouse uPA (20nmol/L) for 24 hours. (C) Cellular PON2 protein expression as determined by Western blot using β-actin monoclonal antibody for assessment of equal loading. The inset shows a representative
blot, and the mean± SD of integrated densitometric data from 6 mice. (D) PON2-associated lactonase activity and (E) PON2 mRNA expression were determined as described in the “Methods” section. Results represent mean ± SD (n=6 mice). *p<0.01 vs. control (without uPA).

Figure 3: The effect of uPA on macrophage oxidative stress.

THP-1 cells were incubated for 24 hours with increasing concentrations of uPA (from 0-60nmol/L). (A) Cellular lipid peroxides content was determined as described in the “Methods” section. (B) Cellular oxidative stress was measured by incubating cells with 10 µM DCFH-DA for 30 minutes at 37°C. Cellular DCF fluorescence was determined by a flow cytometry analysis and expressed as mean fluorescence intensity (MFI) generated by 10,000 cells. (C) THP-1 macrophages were incubated for 24 hours with uPA (20 nml/L) in absence or presence of pomegranate juice (PJ, 12.5, 25 and 50 µmol/L of total polyphenols), DPI (2.5, 5, and 10 µmol/L) or apocynin (25, 50, and 100 µmol/L) and cellular oxidative stress was measured by DCFH oxidation using FACS as described in (B). Results are expressed as mean ± SD (n=3 separate experiments), *p<0.01 vs. control (without uPA).
**Figure 4:** The effect of uPA on NADPH oxidase activation and macrophage-mediated LDL oxidation.

THP-1 cells were incubated for 24 hours with increasing concentrations of uPA (from 0-60nmol/L). (A) Superoxide anion release was measured directly in the medium after incubation of cells with HBSS containing acetyl ferricytochrome C (150 µmol/L) and PMA (100 µg/L, 25), for 20 minutes at 37°C. (B) Gene expression of p47\textit{phox}: ß-Actin cDNA product was used as internal standard to normalize p47phox mRNA levels. C) Macrophage-mediated oxidation of LDL was measured as described in "Methods". Results are expressed as mean ± SD (n=3 separate experiments), *p<0.01 vs. control (without uPA).

**Figure 5:** The effect of uPA on macrophage oxidative stress and PON2 expression in mouse peritoneal macrophages (MPM) from p47\textit{phox} deficient (p47\textit{phox} \textsuperscript{-/-}) mice in comparison to control C57Bl/6 mice. MPM were incubated for 24 hours with increasing concentrations of uPA. (A) Cellular oxidative stress was measured by the DCFH oxidation using FACS, as described in Figure 1B. (B) mRNA expression of p47\textit{phox} in MPM from the control mice and from the p47\textit{phox} \textsuperscript{-/-} mice was determined as described in Figure 2B. (C) PON2 lactonase activity was measured as described in Figure 1F. (D) PON2 mRNA expression was determined by semi quantitative RT-PCR amplification. The housekeeping gene ß-actin was used as a control. The
figures show the mean±SD of integrated densitometric data from 3 separate experiments, each of which included 3 mice, and the inserts show a representative illustration. *p<0.01 vs. control (without uPA).

**Figure 6**: The effect of uPA on macrophage oxidative stress in MPM derived from PON2 deficient (PON2<sup>−/−</sup>) mice in comparison to C57Bl/6 littermate mice.

MPM harvested from PON2<sup>−/−</sup> mice and C57Bl/6 mice were incubated for 24 hours with mouse uPA (20nmol/L). (A) MPM oxidative stress was measured by the DCFH oxidation assay using FACS as described in Figure 1B. Right panel shows a representative illustration. (B) Superoxide anion release was measured directly in the medium as described in Figure 2A. C) Macrophage-mediated oxidation of LDL was measured as described in Figure 2C. Results are expressed as mean ± SD (n=3 separate experiments, each of which included 3 mice). *p<0.01, #p<0.02 vs. control (without uPA).
References


Table I. The effect of aprotinin on plasmin generation and on uPA-induced macrophage PON2 activity.

<table>
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<th>Plasmin generation (OD 405nm)</th>
<th>PON lactonase activity (U/mg cell protein)</th>
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<tr>
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<td>+ aprotinin (1 µmol/L)</td>
<td>+ aprotinin (1 µmol/L)</td>
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<tr>
<td>Control</td>
<td>0.193±0.016</td>
<td>0.034±0.006</td>
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<td>2.10±0.18</td>
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<td>2.14±0.21</td>
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<tr>
<td>+uPA (20nmol/L)</td>
<td>0.499±0.019</td>
<td>0.035±0.006</td>
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<tr>
<td></td>
<td></td>
<td>4.02±0.30</td>
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<td>3.87±0.27</td>
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THP-1 cells were incubated for 24 hours at 37°C without (Control) or with uPA (20nmol/L) in serum-free media containing 0.2% BSA, in absence or presence of aprotinin (1µmol/L). Then, plasmin generation was determined in the conditioned media incubated with the chromogenic substrate of plasmin (S-2251) for 180 minutes, as described in "Methods". Macrophage PON lactonase activity was determined as described in "Methods". Results represent mean ± SD of three experiments.
Legend to Figures (for online publication only)

**Figure I:** Plasmin calibration curve: Concentration and Time study.
Increasing concentrations of plasmin were incubated with 100µg/mL of S-2251 for increasing periods of time. Absorbance at 405nm was then recorded.

**Figure II:** uPA activity on plasminogen: Concentration study. Plasminogen (15µg/mL) was incubated at 37ºC for 20 minutes with increasing concentrations of human uPA in absence (control) or presence of amiloride (2.5µmol/L). Then, 100µg/mL of the plasmin substrate S-2251 was added and the optical absorbance obtained at 405 nm was recorded immediately and after 3 minutes. Generated plasmin was quantified using a calibration curve performed with purified plasmin. Results represent mean±SD of 3 separate experiments.

**Figure III:** Plasmin generation in conditioned media (CM). THP-1 cells were grown for 24 hours in media containing BSA 0.2% alone or with the additions of the followings: human uPA (20nmol/L), plasmin (1µg/mL), plasminogen (1mg/mL). In one experimental set the cells were preincubated for 30 minutes with mouse anti-human uPA-R blocking antibody (anti-CD87, 10µg/mL) before the addition of human uPA (20nmol/L). Then, conditioned media (CM)
was collected and incubated with 100 µg/mL S-2251 at 37°C for increasing periods of time, after which the OD at 405 was measured and recorded. Plasmin generation is expressed as the absorbance at 405 nm.

**Figure IV:** THP-1 cells were incubated for 24 hours at 37°C without (Control) or with uPA (20nmol/L) in serum-free media containing 0.2% BSA. One experimental set was incubated in media containing 5% FCS. All other compounds were added in serum-free media at the following concentrations: ATF-uPA – 20nmol/L, amiloride – 2.5µmol/L, pomegranate juice PJ) – 50mmol/L of polyphenols, DPI - 5 µmol/L, and apocynin- 100 µmol/L. Then, plasmin generation was determined in the conditioned media after incubation with the chromogenic substrate of plasmin (S-2251) for 30 minutes, as described in "Methods". Results represent mean ± SD (n=3 separate experiments).
Figure I

![Graph showing absorbance (OD at 405nm) over time of incubation with S-2251 (minutes). The graph includes lines for different concentrations of plasmin (2 ug, 5 ug, 10 ug, 15 ug, 20 ug) and shows the absorbance values at various time points up to 200 minutes.]
Figure II

Plasmin concentration (μg/mL) vs. uPA concentration (nmol/L) for control and + Amiloride (2.5 μmol/L) treatments.
Figure III

Absorbance (OD at 405nm)

Time of incubation with S-2251 (minutes)

- uPA (20 nmol/L)
- BSA (0.2%)
- uPA + anti CD87
- Plasminogen (1 μg/mL)
- Plasmin (1 μg/mL)
Figure IV

![Graph showing Plasmin (µg/mL) for different treatments. The x-axis represents various substances: FCS, BSA, ATF-uPA, Amiloride, Pomegranate Juice, DPI, and Apocynin. The y-axis shows the concentration in µg/mL. Asterisks indicate significant differences between control and + uPA conditions.](graph_url)