Involvement of reactive oxygen species on gentamicin-induced mesangial cell activation

Carlos Martínez-Salgado, Nélida Eleno, Paula Tavares, Alicia Rodríguez-Barbero, Javier García-Criado, Juan P. Bolaños, and José M. López-Novoa

Departamento de Fisiología y Farmacología and Instituto "Reina Sofía" de Investigación Nefrológica, Universidad de Salamanca, Salamanca, Spain; Departamento de Farmacología e Terapeutica Experimental, Universidade de Coimbra, Portugal; and Departamento de Cirugía and Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, Salamanca, Spain

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Background. Reactive oxygen species (ROS) have been shown to be involved in the reduction of glomerular filtration rate observed after gentamicin (Genta) treatment in vivo, a phenomenon directly related with mesangial cell (MC) contraction. Our previous study reported that Genta induces concentration-dependent MC contraction and proliferation in vitro.

Methods. To study the possible mediation of ROS in the effect of Genta, ROS production was measured in primary cultures of rat MC stimulated with Genta (10^{-5} mol/L). In addition, the MC response to Genta in the presence of the ROS scavengers superoxide dismutase (SOD) and catalase (CAT) was studied. MC activation and O_2^- production were studied in the presence of an inhibitor of the NADP(H) oxidase, diphenylene iodinium (DPI), and in the presence of L-NAME, an inhibitor of nitric oxide synthases (NOS). Finally, the effects of Genta on SOD activity and mRNA expression were examined.

Results. Genta (10^{-5} mol/L) induced an increase in O_2^- production and SOD activity that was neither accompanied by an elevation in cytosolic Cu/Zn-SOD mRNA expression nor by H_2O_2 accumulation. Genta induced MC contraction and proliferation that were inhibited by SOD plus CAT. Both the extracellular and intracellular ROS donor systems, xantine+xantine oxidase (X+XO) and dimethoxinaphtoquinone (DMNQ), respectively, also stimulated MC contraction and proliferation. Genta-induced MC activation and O_2^- production were inhibited by DPI. Genta-induced O_2^- production was inhibited by L-NAME. Furthermore, Genta did not induce detectable changes in membrane fluidity and lipid peroxidation.

Conclusions. These results strongly suggest that an oxidative-mediated pathway exists in Genta-induced MC activation. A portion of the production of O_2^- may be due to NADP(H) oxidase and NOS activation. The amount of ROS produced, rather than having a toxic effect, might play a role as a mediator of Genta-induced MC activation

One of the major side effects of gentamicin (Genta) treatment is nephrotoxicity. The best known effect of this aminoglycoside in the kidney is tubular cell toxicity, but chronic treatment with Genta also modifies glomerular hemodynamics as it reduces renal blood flow (RBF) and the glomerular filtration rate (GFR) without apparent glomerular damage [1]. GFR reduction has been attributed to a decline either in glomerular plasma flow or in the ultrafiltration coefficient (K_f), or both [2–4]. K_f regulation depends mainly on the activity of intraglomerular mesangial cells (MC) because they possess the capacity to contract or relax, thus modifying the ultrafiltration surface; this dynamic phenomenon is highly regulated by numerous vasoactive substances and modified by others [5]. The reduction of the K_f observed after Genta treatment in vivo has been attributed to a mesangial contractile response [2, 4].

There is increasing evidence suggesting that Genta-induced glomerular dysfunction in vivo is mediated by reactive oxygen species (ROS), since administration of antioxidants attenuated the reduction in GFR [6–8]. Superoxide dismutase (SOD) administration in rats treated with gentamicin was associated with a marked increase in RBF, suggesting that O_2^- must be responsible for renal vasoconstriction induced by Genta in vivo [7]. In vitro experiments have shown that Genta enhances ROS production and that renal cortical mitochondria were the source of ROS [9]. The same authors showed that ROS could be responsible for proximal tubular necrosis and acute renal failure caused by Genta in vivo [10]. Administration of antioxidants is beneficial in arresting renal damage produced by endotoxin plus Genta [8]. Additionally, an elevated production of ROS may augment the renal susceptibility to Genta observed in obstructive jaundice [11]. In summary, an enhanced production of ROS has been demonstrated to be involved in the glomerular and tubular alterations characteristic of acute renal failure induced by Genta.

Our previous studies on the glomerular effects of Genta in vitro (isolated glomeruli and cultured MCs) have demonstrated that Genta induces a dose-dependent MC contraction and proliferation [12–14]. In addition, we have demonstrated that ROS directly stimulate MC contraction [15]. Other authors have shown that O_2^- is produced in MCs stimulated with angiotensin II [16] and that ROS are involved in angiotensin II-induced smooth muscle cell proliferation [17, 18].

The aim of the present study is to assess if an increase in ROS production could be involved in the mechanism of action of Genta on MC activation (contraction and proliferation). As an increased production of ROS may induce membrane peroxidation, which results in the loss of membrane integrity and function [6], we also measured the changes in membrane fluidity and lipid peroxidation induced by Genta in vitro.

METHODS

Materials and reagents

Fluorescent probes TMA-diphenylhexatriene (TMA-DPH), diphenylhexatriene (DPH), dichloro-dihydro-fluoresceine (DCHF) and dihydro-rhodamine (DHRh) were purchased from Molecular Probes (Eugene, OR, USA). The sterile plastic material used in cell culture was obtained from Nunc (Roskilde, Denmark). Xanthine (X), xanthine oxidase (XO), superoxide dismutase (SOD), catalase (CAT), dymetoxynaphtoquinone (DMNQ), diphenylene iodinium (DPI), L-nitro-arginine methyl ester (L-NAME), phenylmethylsulfonyl fluoride (PMSF), Nonidet-P40 (NP40), sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA) and cytochrome C were obtained from Sigma Química (Madrid, Spain). [³H]thymidine was purchased from New England Nuclear (Bad Homburg, Germany). Culture medium RPMI 1640 was from Gibco Labs (Barcelona, Spain) and fetal calf serum (FCS) from Whittaker Labs (Barcelona, Spain). A kit to measure LDH activity was obtained from Roche Diagnostics (Manheim, Germany). All other reagents used were of analytical grade and obtained from Sigma Química, Probus (Madrid, Spain), and Merck (Madrid, Spain).

Mesangial cell culture

Primary cultures of MCs were obtained from 150 g female Wistar rats, and glomeruli were isolated by successive mechanical sieving as previously described [12]. Studies were performed in MCs from the first to the second passages. Rats were bred in the animal house of the Edificio Departamental (University of Salamanca, Spain). Animals were treated following the Recommendations from the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals stated in the international regulations and in the following European and national institutions: Conseil de l'Europe (published in the Official Daily N. L358/1-358/6, 18th December 1986), and Spanish Government (published in Boletín Oficial del Estado N. 67, pp. 8509-8512, 18th March 1988, and Boletín Oficial del Estado N. 256, pp. 31349-31362, 28th October 1990).

Obtaining mesangial cell suspensions and membrane-enriched fractions

Mesangial cells were harvested from the surface of culture bottles by treatment with 0.05% trypsin and 0.02% EDTA, washed twice with phosphate buffered saline (PBS; 2.6 mmol/L PO_4H_2K, 4.1 mmol/L PO_4HNa_2, 0.81% NaCl, pH 7.4) and suspended in an appropriate buffer solution. Membrane-enriched protein fractions were obtained from MC suspensions. Cells were lysed in 140 mmol/L NaCl, 10 mmol/L EDTA, 10% glycerol, 20 mmol/L Tris pH 8, 100 U/mL aprotinin, 2 mmol/L PMSF, 60 μg/mL soybean trypsin inhibitor and 1% NP40 at 4°C for 15 minutes. The cell lysates were centrifuged at $5000 \times g$ for 18 minutes at 4°C. The supernatants were centrifuged again at $18000 \times g$ for 45 minutes at 4°C. The pellets were collected and suspended in an appropriate buffer for analytical determinations. Protein content was determined by the Bradford method [19].

Cell viability was measured in mesangial cells incubated with DPI (10^{-5} mol/L), the only compound that we did not use before, by measuring lactate dehydrogenase (LDH) in the culture medium with a commercial kit in a Hitachi 917 spectrophotometer (Ibaragi, Japan).

Determination of planar cell-surface area

Direct observation of MCs grown in conventional plastic culture plates was carried out at room temperature under phase contrast with an inverted Nikon photomicroscope (Tokyo, Japan) using a video camera (Hitachi KP 110) and a Hitachi monitor. Cells were incubated with either Genta 10^{-5} mol/L, X (0.2 mmol/L) + XO (2 mU/mL), or DMNQ (3 μmol/L) and serial photographs of the cells were taken prior to and at several time points post-treatment using an on-line video printer (Sony UP-910; Sony Corp., Tokyo, Japan) [12]. In some culture plates, cells were preincubated for 10 minutes with SOD (15 U/mL) and CAT (80 U/mL), or for 30 minutes with DPI (10^{-5} mol/L) or L-NAME (10^{-5} mol/L) prior to the addition of Genta. Planar cell-surface area (PCSA) was determined by computerized image analysis (IBAS II image analyzer system; Kontron Medical, Eching, Germany). The actual area was calculated after correcting for microscope and photographic magnification. Five to ten cells were analyzed per photograph. In every experimental set the cells were from the same culture.

Proliferation studies

Cell proliferation was measured by both [³H-methyl]-thymidine incorporation into DNA and number of viable

cells. For this purpose, cells were sub-cultured by treatment with 0.05% trypsin and 0.02% EDTA, and plated in 6×4 well plates (Nunc). Experiments were performed on passage one with cells approaching confluence in order to avoid cell dedifferentiation. [³H-methyl]thymidine incorporation into DNA was carried out following the method previously described [12]. Forty-eight hours before starting the experiments, cells were left in culture medium provided with 0.5% FCS; at this time the cells incorporated a minimal amount of [³H-methyl]thymidine, indicating a quiescent state. Then, cells were reactivated by exposure to the same culture medium supplemented with 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium in the presence of Genta (10^{-5} mol/L), X (0.2 mmol/L) + XO (2 mU/mL), or DMNQ (3 µmol/L). In some culture plates, SOD (15 U/mL), CAT (80 U/mL) or DPI (10^{-5} mol/L) was added just before Genta.

The number of cells was measured using a colorimetric method previously described [20]. In brief, cells sub-cultured to sub-confluence in 24 well plates were incubated for 24 hours under the experimental conditions described earlier. Then, cells were fixed with 1% glutaraldehyde for 10 minutes, and washed twice with Hank's solution. Cellular nuclei were dyed by incubating the cells for 30 minutes in a 1% crystal violet solution. Wells were washed exhaustively with distilled water, and left overnight to dry. Finally, 2 mL of 10% acetic acid were added to each well. Optical density at 595 nm was proportional to the number of viable cells in each well.

Detection of H₂O₂ production

Dihydro-rhodamine (DHRh) can be oxidized to the fluorescent product rhodamine by endogenous peroxidases in the presence of H_2O_2 [21]. In order to prevent DHRh oxidation, preparation of the dilution was carried out under an atmosphere of nitrogen. In one set of experiments, cell suspensions were incubated for 60 minutes at 37°C in oxygenated PBS containing DHRh (final concentration 2 µmol/L). Cells were centrifuged at 1800 rpm for three minutes and resuspended in 1.7 mL of the same buffer. Genta (10^{-5} mol/L) was added to the cell suspension while recording the fluorescence. Changes in fluorescence were measured under continuous stirring using a fluorescence spectrophotometer equipped with a thermostatically-controlled cuvette holder (Perkin-Elmer LS-50, Madrid, Spain) at an excitation and emission wavelengths of 488 and 525 nm, respectively. In some experiments, X (0.2 mmol/L) and XO (0.002 U/mL) were added to the cell suspension instead of Genta. DHRh oxidation control was performed at the end of each experiment by adding H_2O_2 (0.1%). The fluorescent signal was registered as a function of the time. Fluorescence obtained was related to the calibration curve performed with increasing concentrations of H_2O_2 (0.0002 to 0.1%). SOD (15 U/mL) and CAT (80 U/mL) were used to ensure the maximal production of H_2O_2 and to measure specific oxidation of the probe by peroxidases, respectively.

Production of intracellular H_2O_2 by MCs also was measured by using the fluorescent probe DCHF [22]. First, the diacetate form of the molecule (DCHF-DAs) diffused readily to the intracellular compartment where it was desacetylated to the non-membrane–permeable DCHF. Then, during the cellular production of H_2O_2, DCHF was oxidized, and emitted a fluorescent signal. The method was essentially the same as that described for calcium [23], with two main differences: MCs were loaded with 20 µmol/L DCHF-DA, and excitation and emission wavelengths were 488 nm and 525 nm, respectively

Detection of superoxide anion (O_2^-) production

Mesangial cells were incubated in the presence of 10^{-5} mol/L Genta for one to 24 hours. Cells were raised from the culture bottles as described earlier. Resuspended cells were sonicated and centrifuged (15 min, $16000 \times g$). The supernatant was collected and proteins measured by the method of Bradford [19]. The protein sample obtained from one culture bottle (80 cm²) was used for each determination. Superoxide anion production was detected by a modification of the technique based in the specific reduction of cytochrome C by O_2^- in the soluble fraction of cells obtained after sonication [24]. Briefly, cytochrome C (75 µmol/L), protein sample and PBS containing EDTA 0.1 mmol/L were added to a final volume of 250 µL in each well. The specific reduction of cytochrome induced by O_2^- was calculated by the difference in reduction in the presence and absence of SOD (130 U). The slope of spectrophotometric change was recorded during one minute in a spectrophotometer at a wavelength of 550 nm, at 25°C. Results are expressed as nmol O_2^-/mg protein/min.

In some experiments, O_2^- production was determined in MCs preincubated with DPI (10^{-5} mol/L) and/or L-NAME (10^{-5} mol/L) prior to the incubation with Genta (10^{-5} mol/L, 24 hours). Zymosan (1 mg/mL) was used as a positive control [25].

SOD activity assay

A time-course (1 to 24 hours) of SOD activity was determined in mesangial cells treated with Genta 10^{-5} mol/L or zymosan (1 mg/mL) according to the method of Misra and Fridovich [26]. The soluble fraction of cultured MCs was obtained as previously described for the determination of O_2^- production. The method is based in the inhibition by SOD of the spontaneous oxidation of epinephrine in a basic medium, which is measured in a spectrophometer at 480 nm. Briefly, epinephrine (10^{-2} mol/L) and protein sample were added to a NaHCO₃-Na₂CO₃ buffer (0.03 mol/L, pH 10.2) to a final volume of 250 µL. Maximal epinephrine autoxidation is measured in the absence of protein sample. One unit (U) of enzymatic activity is the amount of SOD necessary to

inhibit the epinephrine autoxidation by 50%. Results are expressed in U/mg protein.

Determination of membrane fluidity

Changes in membrane fluidity were assessed in whole cells and in a membrane-enriched fraction of MCs. This method is based on the measurement of fluorescence stationary anisotropy evoked by the fluorescent hydrophobic probe DPH and its cationic trimethylammonium derivative (TMA-DPH) [27]. TMA-DPH is supposed to be anchored to the surface of the membrane by its electric charge, and is used to monitor fluidity of the outer layer of the plasma membrane. DPH is localized within the hydrophobic membrane core and provides information about fluidity of this region. We used TMA-DPH for determinations in whole cells and membrane-enriched fractions, whereas DPH was used to measure changes in whole cells only. Samples were illuminated with linear polarized light (360 nm and 363 nm excitation wavelength for TMA-DPH and DPH, respectively), and the parallel and perpendicular components of the emitted light intensity were measured at a wavelength of 428 nm and 427 nm for TMA-DPH and DPH, respectively. Scattered light was corrected automatically in a Perkin-Elmer LS-50 spectrofluorometer. Previously, either the number of whole cells or the amount of protein used in each sample was adjusted in order to obtain a basal value of anisotropy equal to the theoretically calculated anisotropy [28]. Cellular suspensions or membrane-enriched protein fractions (2 mL containing 150,000 cells or 18 μg of protein) were incubated with 1 μmol/L probe at 37°C under gentle stirring. After 15 minutes of stabilization, Genta was added and changes in fluorescence anisotropy were recorded at different times (0 to 70 min). Changes in anisotropy were also measured in suspensions of MCs treated with Genta during 24 hours in the culture bottle.

In addition to studies on mesangial cells, we also studied the effects of gentamicin on membrane fluidity and lipid peroxidation in proximal tubular membranes. Female Wistar rats were treated with Genta [100 mg/kg body weight (BW)/day, SC in 0.5 mL of saline every day for 3 days]. The kidneys were removed, and in renal cortical tissue, purified brush border and basolateral cortical membranes (BBM and BLM, respectively) were obtained by differential centrifugation and assessed by the enrichment in Na,K-ATPase, glucose 6-phosphatase, succinate dehydrogenase γ-glutamyl-transpeptidase and alkaline phosphatase as previously described [29]. Membrane suspensions were incubated with the solution of the probe in the dark (30 min, 37°C). Changes in fluorescence anisotropy were measured as explained earlier in this article.

Determination of membrane lipid peroxidation

This method is based in a thiobarbituric acid (TBA) reaction with lipid peroxides produced during membrane lipid peroxidation [22]. The oxidation of membrane lipids is likely to result in the formation of peroxidation degradation products such as the highly reactive compound malondialdehyde (MDA), leading to cross-linking reactions of the lipid-lipid and lipid-protein types, and thereby causing rigidity of the membrane and decreasing the fluidity. After incubating MCs with 10^{-5} mol/L Genta during one and 24 hours, samples enriched in plasma membranes were obtained as reported earlier in this article. After deproteinization, a volume of 400 μL of TBA 1%—diluted in water:glacial acetic acid, 1:1— was added to the samples. The mixture was boiled during 15 minutes, the reaction was stopped on ice (4°C) and centrifuged at $10000 \times g$ for 10 minutes, at 4°C. Optical density of the supernatants was measured at 532 nm and values calculated by interpolation in a calibration curve performed with malondialdehyde (0 to 10 nmol/mL).

We also determined the tiobarbituric reacting substances (TBARS) in BBM and BLM of renal cortical cells of rats subjected to Genta treatment in vivo, as described earlier.

RNA isolation and Northern blot analysis of SOD

Total RNA was isolated from MCs (plated in 28.2 cm^2 wells) by the guanidium thyocyanate method [30], and quantified spectrophotometrically at 260 nm. RNA was loaded (10 μg/lane) on a 1% (wt/vol) agarose-formaldehyde gel. After separation by electrophoresis, the RNA was transferred onto a GeneScreen Plus membrane (NEN Life Science, Boston, MA, USA) and cross-linked with ultraviolet irradiation (UV Stratalinker, Mod. 2400; Genetic Res. Instruments, Essex, UK). Membranes were hydrated with 2× saline solution concentrated (20 × SSC = 3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7) and preincubated at 60°C in hybridization solution [1% (wt/vol) SDS, 1 mol/L NaCl, 10% (wt/vol) dextran sulfate]. After 10 minutes, the membranes were incubated for 18 hours at 60°C in the same solution containing ^{32}P-labeled Cu/Zn-SOD cDNA, Mn-SOD cDNA or cyclophilin cDNA probes. The rat Cu/Zn-SOD 0.6 kb and Mn-SOD 1.4 kb cDNAs clones were generous gifts from Dr. Ye-Shih Ho (Wayne State University, USA) and were digested from pBluescript KS with EcoRI. A 0.7 kb BamHI cDNA fragment of rat cyclophilin gene, isolated from pBSK+ vector (generously donated by Dr. Dionisio Martín-Zanca, Universidad de Salamanca, Spain) was used as a control of the amount of total RNA loaded in each lane. Approximately 25 ng of the fragment was labeled using a Boëhringer random-primed labeling kit with 2 μL of [a-^{32}P]dCTP (2 μCi; 3000 Ci/mmol), 3 μL of a mixture of dATP, dGTP and dTTP (0.5 mmol/L each), 2 μL of hexanucleotide mix and 1 μL (2 U) of Klenow enzyme in a total volume of 20 μL for 30 minutes at 37°C. The ^{32}P-labeled cDNA was purified in a Sepharose column and hybridized with the membrane for 18

hours at 60°C. After hybridization, the membrane was washed once for five minutes at 60°C in 2 × SSC, twice for 30 minutes at 60°C in 1 × SSC provided with 0.5% (wt/vol) SDS, and once for 60 minutes at room temperature in 0.1 × SSC provided with 0.1% (wt/vol) SDS. Membranes were exposed to Kodak XAR-5 film for two to three days at −70°C and autoradiograms were subsequently scanned.

Statistical analysis

Data are expressed as mean ± standard error of the mean (X ± SEM). Comparison of means was performed by one or two way analysis of variance (ANOVA). Statistical differences between groups were assessed by the Scheffe method.

RESULTS

Effect of ROS scavengers on Genta-induced MC contraction and proliferation

We explored the possibility that Genta-induced MC contraction could be blocked by ROS scavengers and found that Genta (10^{-5} mol/L) induced a time-dependent reduction in mesangial PCSA. Preincubation of MCs with SOD (15 U/mL) plus CAT (80 U/mL) blunted the Genta-induced reduction in PCSA. In addition, SOD and CAT themselves did not induce any change per se in PCSA (Fig. 1A).

The next experiments demonstrated that ROS donors could simulate the effect of Genta on MC contraction. The extracellular and intracellular O_2^- donor systems, X (0.2 mmol/L) + XO (2 mU/mL) or DMNQ (3 μmol/L), respectively, induced a time-dependent reduction in PCSA (Fig. 1B). No changes in PCSA were observed in MCs incubated with culture media alone (control).

To demonstrate whether the effects of ROS scavengers and ROS donors on MC contraction also could be observed in MC proliferation, we measured [^{3}H-methyl]-thymidine incorporation into the DNA. Genta (10^{-5} mol/L, 24 hours) increased thymidine incorporation by approximately threefold in quiescent MCs (Fig. 2A). In addition, 10^{-5} mol/L Genta increased the number of MCs 1.8 times with respect to control conditions (Fig. 3A). Both effects were inhibited by preincubation with SOD (15 U/mL) plus CAT (80 U/mL) prior to the addition of Genta. Incubation with either X+XO or DMNQ also increased the [^{3}H-methyl]-thymidine incorporation into DNA, and viable cell number (Figs. 2B and 3B). The combination SOD + CAT by itself did not induce any significant change on [^{3}H-methyl]-thymidine incorporation into DNA or cell number. SOD alone inhibited contraction but did not inhibit proliferation in gentamicin-stimulated mesangial cells (Fig. 4).

To discard the interferences of ROS with the colorimetric assay to measure cell number, we performed an
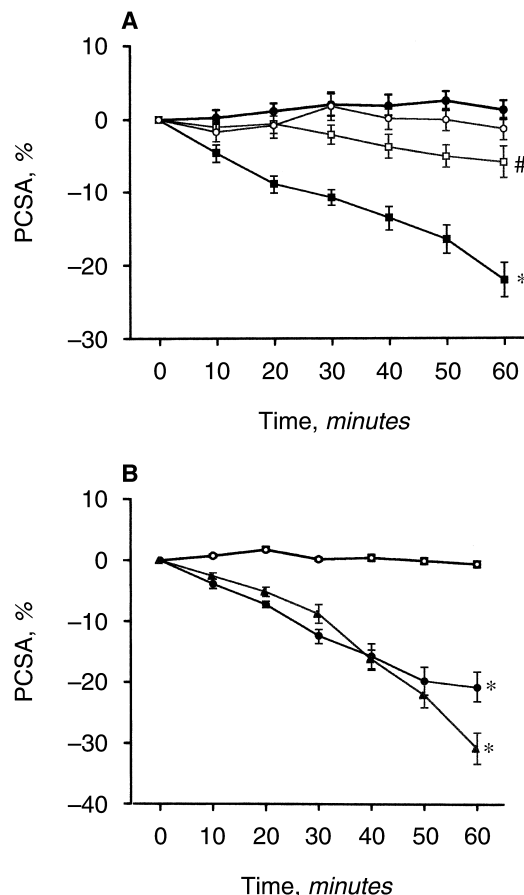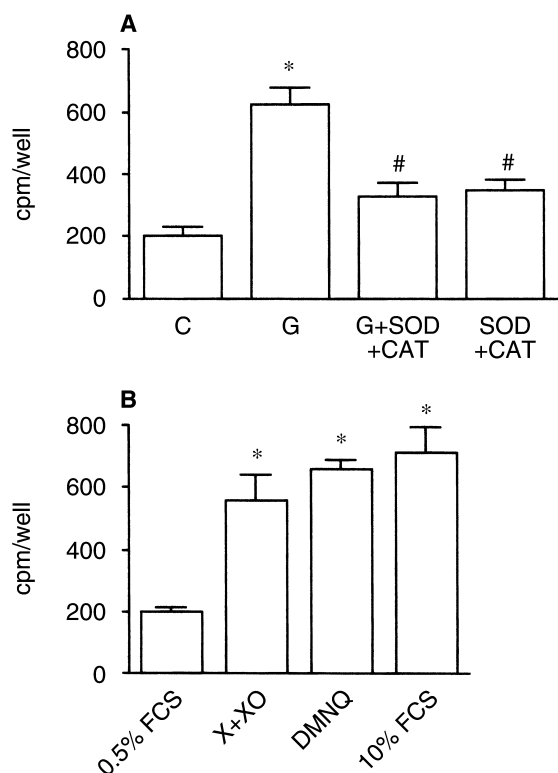

Fig. 1. Effects of gentamicin (Genta) either in the presence or the absence of reactive oxygen species (ROS) scavengers (*A*), and effect of ROS donors (*B*), on planar cell surface area (PCSA) in cultured mesangial cells (MCs). Data are means ± SEM (as % of basal PCSA measured at time 0) of 5 experiments with 5 to 10 cells measured in each one. Abbreviations are: C, non-stimulated cells in control conditions; G, 10^{-5} mol/L Genta; SOD, 15 U/mL superoxide dismutase; CAT, 80 U/mL catalase; X+XO, 0.2 mol/L xanthine + 2 mU/mL xanthine oxidase; DMNQ, dimethoxinaphtoquinone 3 μmol/L. Symbols in A are: (●) C; (■) G; (□) G+SOD+CAT; (○) SOD+CAT. Symbols in B are: (○) C; (●) X+XO; (▲) DMNQ. Statistically significant differences are: *$P < 0.01$, with respect to cells incubated in control conditions; #$P < 0.01$, with respect to cells incubated with Genta.

experiment in which mesangial cells were incubated with Genta, with X+XO and with both stimuli during one hour, enough time to produce ROS but not proliferation. The absorbance of the crystal violet gave similar values of cell number in all groups, and the same than in control cells (0.5% FCS), demonstrating the absence of interferences between ROS and this colorimetric assay (Table 1).

Effect of NADP(H) oxidase inhibition on Genta-induced MC contraction and proliferation

As it has been demonstrated that activated smooth muscle cells produce ROS derived from NADP(H) oxidase activity [18], we assessed the effect of NADP(H) inhibition on Genta-stimulated MC contraction and pro-

Fig. 2. Effects of Genta either in the presence or the absence of reactive oxygen species (ROS) scavengers (*A*), and effect of ROS donors (*B*), on [³H-methyl]thymidine incorporation into DNA in cultured MCs. Data are means ± SEM of 6 to 10 experiments, each performed in triplicate. Abbreviations are: C, non-stimulated cells in control conditions (0.5% FCS); G, 10^{-5} mol/L Genta; SOD, 15 U/mL superoxide dismutase; CAT, 80 U/mL catalase; X+XO, 0.2 mmol/L xanthine + 2 mU/mL xanthine oxidase; DMNQ, dimethoxinaphtoquinone 3 μmol/L; FCS, fetal calf serum. Statistically significant differences were: *$P < 0.01$ with respect to cells incubated in control conditions (0.5% FCS); #$P < 0.01$ with respect to cells incubated with Genta.
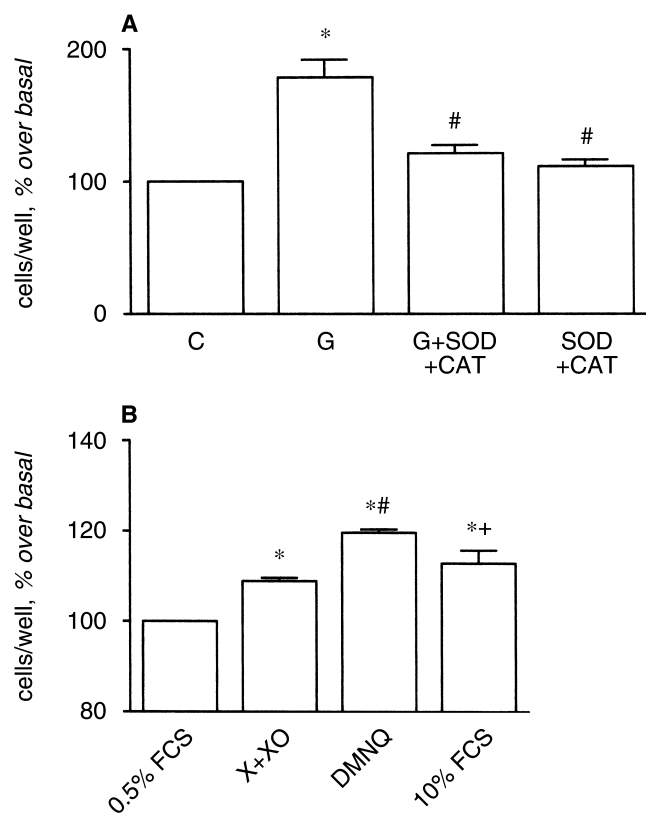
Fig. 3. Effects of Genta either in the presence or the absence of reactive oxygen species (ROS) scavengers (*A*), and effect of ROS donors (*B*), on crystal violet nuclear staining in cultured MCs. Data are means ± SEM (expressed as % of nuclear staining measured in cells incubated in control conditions) of 6 experiments with 6 wells measured in each one. Abbreviations are: C, non-stimulated cells in control conditions (0.5% FCS); G, 10^{-5} mol/L Genta; SOD, 15 U/mL superoxide dismutase; CAT, 80 U/mL catalase. Statistically significant differences are: *$P < 0.01$ with respect to cells incubated in control conditions; #$P < 0.01$ with respect to cells incubated with Genta in (A) or X+XO in (B); +$P < 0.01$ with respect to cells incubated with DMNQ.

liferation. Pretreatment of MC with DPI (10^{-5} mol/L), an inhibitor of the NADP(H)oxidase, reduced the Genta-induced time-dependent reduction in PCSA (Fig. 5A). In addition, DPI (10^{-5} mol/L) inhibited the Genta-induced [³H-methyl]-thymidine incorporation into DNA (Fig. 5B).

As DPI completely abolished the cellular response, we checked for DPI-induced cell toxicity. There was a lack of LDH activity in cells treated during 24 hours with gentamicin, DPI or gentamicin+DPI; moreover, LDH values did not show differences with the control cells (0.5% FCS; Table 2).

Production of O_2^- and H_2O_2 in Genta-stimulated MCs

Increased production of both O_2^- and H_2O_2, have been reported to occur in MCs in response to several stimuli. Thus, we checked which ROS were produced by MCs following incubation with Genta. Significant production of O_2^- in MCs stimulated with 10^{-5} mol/L Genta were observed as early as after one hour of incubation, and this production increased progressively until 24 hours (5.0

times of increase over control cells incubated in 0.5% FCS). After an incubation with zymosan for 24 hours, O_2^- production was 4.8-times higher than in untreated cells (Table 3). Therefore, Genta induced a constantly increased production of O_2^-, an effect that was quantitatively similar to the effect elicited by zymosan (1 mg/mL).

The oxidation of the fluorescent probe DHRh by accumulative concentrations of H_2O_2 (0.0002 to 0.1%) produced a dose-dependent increase in fluorescence. Final addition of CAT reduced the maximal fluorescence signal by about one-third. Changes in fluorescence were not observed in cells incubated with Genta at different times until four hours (results not shown). Moreover, incubation of MCs with H_2O_2 (10^{-4} mol/L) induced a rapid and transient increase in DCHF fluorescence. Incubation with Genta (10^{-5} mol/L) did not induce any change in fluorescence (results not shown). Thus, no significant production of H_2O_2 by MCs incubated with Genta was detected by two methods.
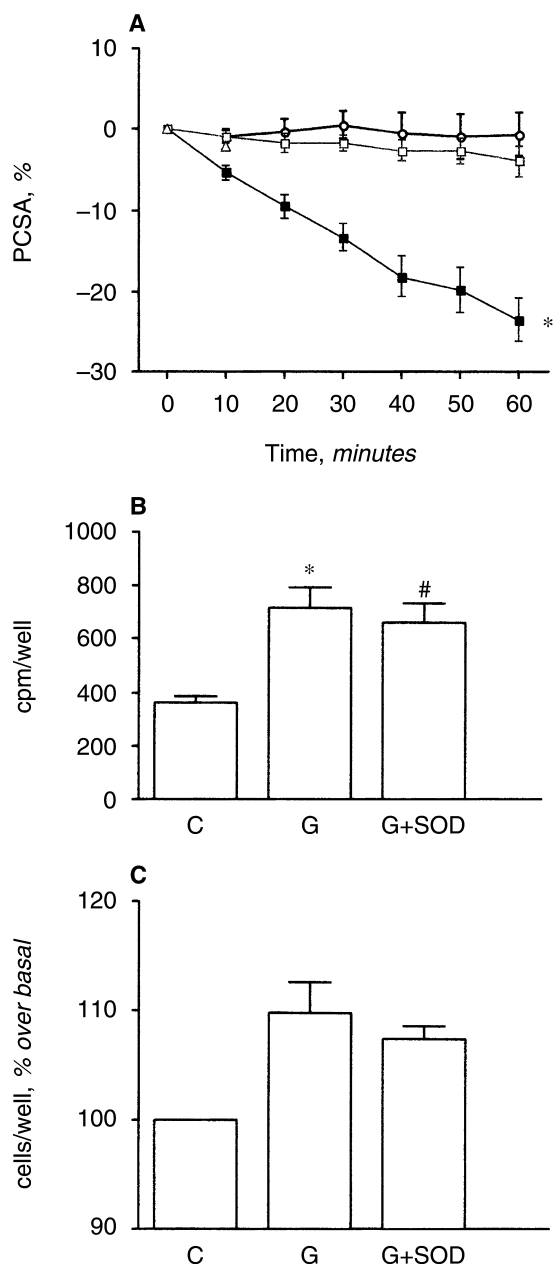
Fig. 4. Effects of Genta either in the presence or the absence of super-oxide dismutase (SOD) on planar cell surface area (PCSA) (0-60 min) (*A*), on ^{3}H-thymidine incorporation into DNA in cultured MCs (*B*), and on crystal violet nuclear staining (*C*) in cultured MCs. Data are means $\pm$ SEM (as % of basal PCSA measured at time 0 of 5 experiments with 5-10 cells measured in each experiment in (A), and expressed as % of nuclear staining measured in cells incubated in control conditions in (C). Abbreviations and symbols are: ($\bigcirc$) C, non-stimulated cells in control conditions; ($\blacksquare$) G, 10^{-5} mol/L Genta; ($\square$) SOD, 15 U/mL super-oxide dismutase. Statistically significant differences were: *$P < 0.01$ with respect to cells incubated in control conditions; #$P < 0.01$ with respect to cells incubated with Genta.

Effect of NADP(H) oxidase inhibition and NOS inhibition on O_2^- production induced by Genta in MCs

To verify whether the inhibitory effect of DPI on mesangial cell activation is mediated by of O_2^-, this anion

Table 1. Lack of interference between reactive oxygen species (ROS) and the colorimetric assay of crystal violet in mesangial cells incubated during one hour in 0.5% fetal calf serum (FCS control, C) with gentamicin (Genta) either in the presence or absence of ROS donor X + XO

	Cells/well
C *0.5% FCS*	77176 ± 1796
Genta *10^{-5} mol/L*	76239 ± 968
(0.2 mmol/L) X + (2 mU/mL) XO	78295 ± 1141
Genta + X/XO	76789 ± 1458

was measured in mesangial cells treated with Genta during 24 hours, either in the presence or in absence of DPI. DPI inhibited O_2^- generation almost completely (Table 4). Moreover, since DPI inhibits flavin-containing enzymes, DPI also would inhibit NOS, which has been previously shown to be activated by Genta [13]. Because NOS can become uncoupled and produce O_2^-, a NOS inhibitor was tested for its effects on O_2^- generation induced by Genta in MCs. L-NAME almost completely inhibited O_2^- generation. This result suggests that NOS effectively can produce O_2^- in MCs stimulated with Genta (Table 4).

SOD activity in Genta-stimulated MC

Mesangial cells stimulated with 10^{-5} mol/L Genta showed an activation of SOD after eight hours of incubation, and this activity remained constant after 24 hours of incubation. Stimulation with Genta or zymosan for 24 hours increased enzymatic activity 2.5-times and 2.4-times higher than in untreated cells, respectively. This means that Genta induced a constant activation of SOD quantitatively similar to that induced by 1 mg/mL zymosan (Table 3).

Expression of SOD mRNA

Probes were used to detect mRNA from both, rat mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD. While mitochondrial Mn-SOD mRNA was undetectable under our conditions, Cu/Zn-SOD mRNA was readily detectable in both control and Genta-treated cells. However, the treatment with Genta did not result in an increased Cu/Zn-SOD mRNA amount (Fig. 6).

Changes in plasma membrane fluidity and lipid peroxidation

As an enhanced production of ROS may induce membrane peroxidation that results in the loss of membrane integrity and function [6], the changes in membrane fluidity and lipid peroxidation induced by Genta were measured.

Membrane fluidity of the outer layer and the core region of the plasma membrane were checked by using TMA-DPH or DPH, respectively. Acute addition of 10^{-5} mol/L Genta to MCs in suspension did not change fluorescence anisotropy of the TMA-DPH incorporated in
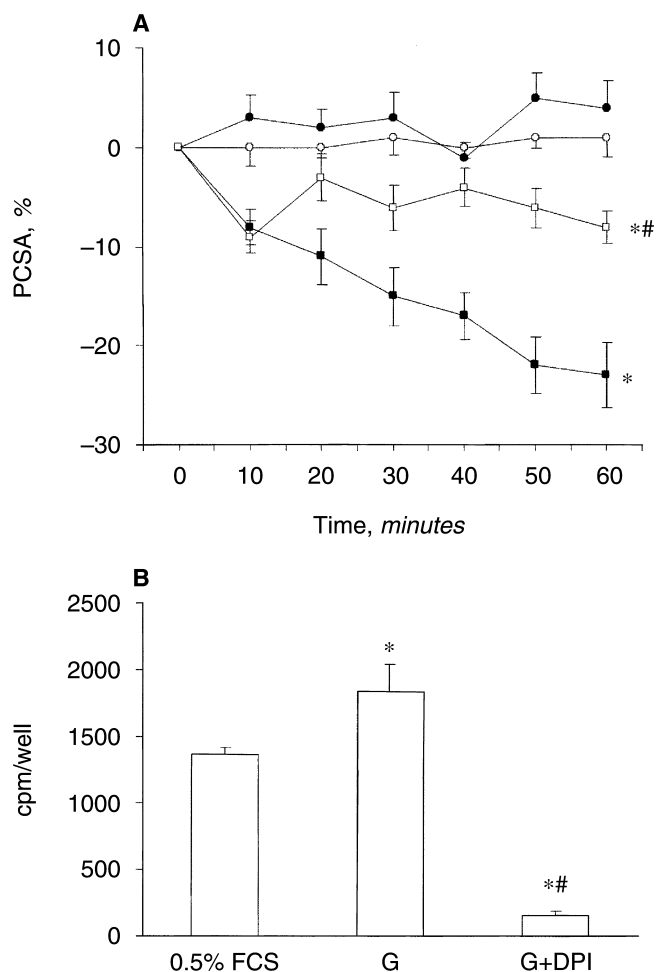
Fig. 5. Effects of Genta either in the presence or in the absence of diphenylene-iodinium (DPI) an inhibitor of the NADP(H) oxidase, on planar cell surface area (PCSA) (0-60 min) (*A*) and, on ³H-thymidine incorporation into DNA in cultured MCs (*B*). Symbols and abbreviations are: (●) C, non-stimulated cells in control conditions; FCS, fetal calf serum; (■) G, 10^{-5} mol/L gentamicin; (○) DPI, 10^{-5} mol/L diphenylene-iodinium; (□) G+DPI. Statistically significant differences were: *$P < 0.05$ with respect to cells incubated in control conditions; #$P < 0.05$ with respect to cells incubated with Genta. (A) Data are mean ± standard error (as % of basal PCSA measured at time 0) of 2 experiments with 5 to 7 cells measured in each one. (B) Data are means ± standard error of 3 experiments, each in triplicate.

Table 2. Lack of cell toxicity measured as lactate dehydrogenase (LDH) released in mesangial cells incubated during 24 hours in 0.5% FCS (control, C) with Genta, either in the presence or absence of DPI

	LDH *U/L*
C *0.5% FCS*	5.4 ± 0.19
DPI *10^{-5} mol/L*	3.2 ± 0.16
Genta *10^{-5} mol/L*	4.1 ± 0.17
Genta + DPI	5.0 ± 0.19

Table 3. Superoxide anion (O_2^-) production and SOD activity in cultured MCs treated with 10^{-5} mol/L Genta from 1 to 24 hours

	O_2^- *nmol/mg protein/min*	SOD *U/mg protein*
C *0.5% FCS*	12.21 ± 1.66	26.6 ± 2.37
Genta		
1 hour	37.17 ± 5.12^b	39.0 ± 5.5
4 hours	42.64 ± 3.86^b	33.3 ± 6.3
8 hours	56.52 ± 4.89^a	47.3 ± 10.1^b
24 hours	60.95 ± 5.05^a	64.3 ± 7.0^b
Zymosan		
1 hour	23.50 ± 2.48	26.0 ± 6.8
4 hours	36.87 ± 9.80	30.6 ± 6.6
8 hours	81.09 ± 9.81^a	112.6 ± 21.3^{bc}
24 hours	58.73 ± 1.21^a	63.3 ± 4.6^b

Production of O_2^- in MCs treated with zymosan (1 mg/mL) was used as positive control. Results are expressed as mean ± SEM of 3 experiments, each in triplicate.
[a] $P < 0.001$ vs. C (0.5% FCS)
[b] $P < 0.05$ vs. C (0.5% FCS)
[c] $P < 0.05$ vs. Genta at the same time

Table 4. Superoxide anion (O_2^-) production in cultured MCs treated with 10^{-5} mol/L Genta during 24 hours either in the presence or absence of DPI and L-NAME

|	O_2^- *nmol/mg protein/min*
C *0.5% FCS*	12.01 ± 1.93
Genta *10^{-5} mol/L*	28.42 ± 1.91^a
Genta *10^{-5} mol/L* + DPI *10^{-5} mol/L*	4.73 ± 1.83^{ab}
Genta *10^{-5} mol/L* + L-NAME *10^{-5} mol/L*	8.66 ± 2.29^{ab}
Genta + DPI + L-NAME	3.61 ± 1.83^{ab}
Zymosan	52.1 ± 7.74^{ab}

Production of O_2^- in MCs treated with zymosan (1 mg/mL) was used as positive control. Results are expressed as mean ± SEM of 3 experiments, each in triplicate.
[a] $P < 0.05$ vs. C (0.5% FCS)
[b] $P < 0.05$ vs. Genta

the membrane (Table 5). Incubation with Genta for longer periods (10 to 70 min) did not induce further changes in fluorescence. Moreover, plated MCs incubated for 24 hours either in the presence or absence of Genta showed similar values in fluorescent anisotropy of the probe TMA-DPH, and identical values of fluorescent anisotropy of the probe DPH (Table 5). Membrane-enriched fractions obtained from MCs incubated for 24 hours either in the presence or absence of Genta showed the same values of fluorescence anisotropy for TMA-DPH (Table 5). Membrane lipid peroxidation, as measured by the production of TBA-reacting products, in MCs treated with Genta for one or 24 hours was negligible (results not shown).

In addition, Genta treatment in vivo increases lipid peroxidation in proximal tubule membranes, as reflected by the increase TBARS (Table 6), and these effects were accompanied by a significant decrease in membrane fluidity because fluorescence polarization (which is inversely related to membrane fluidity), was significantly enhanced using either of the fluorescent probes DPH or TMA-DPH (Table 7). As these probes are positioned in the membrane in the central hydrophobic zone and the external hydrophilic area, respectively, we can deduce that all the membrane is affected by gentamicin treatment. However, these effects were only observed in the BBM, and not in the BLM membrane.
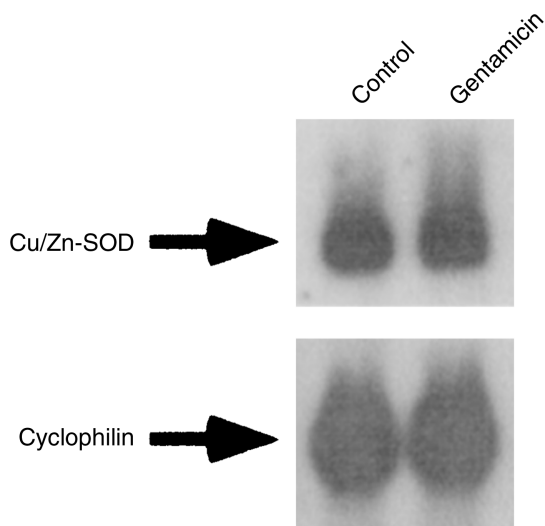
Fig. 6. Northern blot analysis of Cu/Zn-superoxide dismutase (SOD; 0.6 kb) in MCs incubated with 10^{-5} mol/L Genta for 24 hours. Cyclophilin was used as a mRNA loading marker. A representative blot of 2 experiments carried out with cells from different cultures is shown.

DISCUSSION

Several hypotheses have been suggested to elucidate the possible mechanism(s) involved in the tubular and glomerular effects of Genta, and one of the proposed mechanisms includes the production of ROS [6–11, 31]. We have previously shown that Genta induces contraction and proliferation in primary cultures of MCs [12, 14, 23]. We also previously observed that H_2O_2 induces MC and glomerular contraction in vitro [15]. Thus, our current study aimed to validate the hypothesis that Genta-induced MC contraction and proliferation in vitro could be mediated by an increased production of oxygen radicals.

One of the major new findings of this study is that Genta, at the concentration used, induces an acute production of O_2^- in cultured MCs that is maintained after 24 hours of treatment. This increased O_2^- production by MCs is not followed by H_2O_2 accumulation. The absence of H_2O_2 production was confirmed with two different intracellular fluorescent probes, DHRh and DCHF. In our study, no effects of Genta on Cu/Zn–SOD mRNA were observed after 24 hours of incubation. However, there was a time-course activation of SOD, thus demonstrating that Genta regulates the enzyme at this level. The Genta-induced O_2^- production without H_2O_2 accumulation can be explained if a major part of O_2^- generated is transformed in peroxynitrite by a reaction with nitric oxide (NO). We have previously demonstrated an increased NO production in MCs incubated with Genta [13]. Nevertheless, the absence of membrane damage characteristic of this particularly aggressive radical make it difficult to accept this hypothesis.

The mechanism by which Genta-stimulated MCs can produce O_2^- is unknown. A possible origin of O_2^- is the activation of mesangial phospholipase A_2 (PLA$_2$) [32]. We have demonstrated that Genta stimulates calcium-dependent PLA$_2$ activity in cultured MCs [12], and PLA$_2$ activation has been associated with increased O_2^- production [33–35]. In addition, we demonstrated an increased PAF production by MCs after incubation with Genta [14], and it is known that PAF stimulates phospholipase A_2–mediated O_2^- release by macrophages [36]. Moreover, O_2^--mediated iron-catalyzed formation of hydroxyl radicals can rapidly and irreversibly inactivate PAF acetylhydrolase [37], a fact that could contribute to increase PAF production after incubation with Genta.

Evidence suggests that angiotensin II-induced O_2^- formation is mediated by NADP(H) oxidase in vascular smooth muscle cells [18]. The cytochrome b558 alpha subunit p22 (phox), one of the key electron transfer elements of the NADPH oxidase, plays a central role in angiotensin II-induced O_2^- generation by smooth muscle cells [17]. This element has also been described to be present in MCs [38]. Another possible source of O_2^- could be NO synthase (NOS) activity.

The present work demonstrates that DPI, an inhibitor of NADP(H)oxidase, almost completely inhibited O_2^- generation, suggesting that this enzyme could be one of the sources of O_2^- in Genta-stimulated MCs, as it has been shown in angiotensin II-stimulated MCs [16]. However, DPI inhibits flavin-containing enzymes, meaning that DPI also would inhibit NOS, which was shown previously to be activated by Genta [13]. Besides, O_2^- generation has been associated to either endothelial NOS [39] or inducible NOS activation [40], and we have already demonstrated that incubation of rat MCs with Genta induces iNOS expression [13]. Therefore, a NOS inhibitor was tested for its effects on O_2^- generation induced by Genta in MCs. We observed that L-NAME almost completely inhibited O_2^- generation, suggesting that NOS effectively might produce O_2^- in MCs stimulated with Genta.

The reduction in PCSA was used to determine the extent of MC contraction, as our earlier study showed a correlation between PCSA and the phosphorylation of the light myosin chain [15]. The Genta-induced mesangial contraction and proliferation demonstrated in the present study and in previous ones is blunted in the presence of SOD and CAT [12, 14, 23]. This observed inhibition is due to the reduction of oxygen free radicals produced by cells and not to the direct effect of the enzymes alone. The inhibition by ROS scavengers of Genta-induced MC contraction in vitro might explain the improvement of glomerular function produced by the administration of antioxidants in animals treated with Genta [6, 7, 41], and confirms the mediation of ROS in the renal effects of this antibiotic. The fact that SOD alone inhibited MC contraction, but not DNA synthesis or cell number, could mean that O_2^- is involved in the

Table 5. Steady-state fluorescence anisotropy for DPH and TMA-DPH in MCs (whole cells or membrane-enriched fractions) treated with Genta at different times (acute addition, 24 hours of incubation)

| | Whole cells | | | | Membrane-enriched fractions | |
	Control	Genta (acute)	Control	Genta (24 h)	Control	Genta (24 h)
DPH	—	—	0.172 ± 0.003	0.172 ± 0.004	—	—
TMA-DPH	0.225 ± 0.007	0.232 ± 0.009	0.243 ± 0.006	0.235 ± 0.003	0.230 ± 0.005	0.230 ± 0.004

Data are expressed as mean $\pm$ SEM of three experiments with 3 to 8 measurements per sample.

Table 6. Lipid peroxidation (tiobarbituric acid reacting substances, TBARS) in brush border membrane (BBM) and basolateral membrane (BLM) from kidney cortex from control rats and rats treated with gentamicin (100 mg/kg BW/day, sc, during 3 days)

| | BBM | | BLM | |
	Control	Gentamicin	Control	Gentamicin
TBARs *nmol/mg protein*	0.21 ± 0.02	0.33 ± 0.002[a]	0.19 ± 0.03	0.21 ± 0.03

Data are expressed as mean $\pm$ SEM of 16 rats, with two rats pooled per data.
[a] $P < 0.05$ with respect to control conditions

Table 7. Steady-state fluorescence anisotropy for DPH and TMA-DPH in brush border membranes (BBM) and basolateral membranes (BLM) from kidney cortex in control rats and rats treated with Genta (100 mg/kg BW/day, sc, during 3 days)

| | BBM | | BLM | |
	Control	Gentamicin	Control	Gentamicin
DPH	0.210 ± 0.002	0.216 ± 0.002[a]	0.190 ± 0.003	0.193 ± 0.003
TMA-DPH	0.229 ± 0.002	0.240 ± 0.002[a]	0.219 ± 0.002	0.219 ± 0.003

Data are expressed as mean $\pm$ SEM of 16 rats, with two rats pooled per data.
[a] $P < 0.05$ with respect to control conditions

rapid contractile response elicited by Genta in MCs, whereas other mechanisms could be involved in the long-term responses such as cell proliferation.

Diphenylene iodinium inhibited gentamicin-induced mesangial contraction and proliferation, and this effect could be due to the inhibition of O_2^- generated either by NADP(H) oxidase or by NOS activities, or both.

The present work shows that L-NAME inhibited O_2^- generation. However, our earlier study on the effects of NOS inhibition in Genta-induced mesangial contraction and proliferation found no effect with L-NAME [13]. This lack of effect of L-NAME could be explained by the inhibitory effect of NO on Genta-induced mesangial activation, opposite to the stimulatory effect of O_2^- observed in the present experiments. The simultaneous production of NO and O_2^- by NOS in Genta-treated cells might have opposite effects on mesangial activation. In fact, this production of O_2^- by NOS can explain previously published results [13] showing that the addition of L-arginine—which increased NO synthesis—inhibited mesangial activation induced by Genta, whereas addition of L-NAME did not have any effect. We can now explain this lack of effect of L-NAME because of the simultaneous decrease in NO and O_2^- produced by NOS activity.

Our data also show enough evidence that the amount of ROS produced by this concentration of Genta does not damage the cell membrane structure. Genta did not induce any detectable change in lipid peroxidation or in membrane fluidity, either in the outer layer or in the hydrophobic core, of the plasma membrane. As a positive control, renal cortical brush border membrane of rats treated with gentamicin for three days showed marked changes in membrane fluidity and lipid peroxidation. Our results suggest that the amount of ROS produced by this concentration of Genta in MCs might act as a mediator of cellular response [34, 42]. Increasing evidence suggests that ROS may have a role as second messengers involved in signal transduction for cell proliferation in MCs [6], myocytes [41], smooth muscle cells [17, 18], chondrocytes [43] and lymphocytes [44, 45].

In summary, this study shows that ROS are generated in cultured MCs exposed to Genta, and that ROS scavengers inhibit Genta-induced mesangial contraction and proliferation. Genta does not cause any change in fluidity or lipid peroxidation in the plasma membrane. Thus, we propose that Genta causes MC activation at least in part via the generation of O_2^-.

Reprint requests to J.M. López-Novoa, Ph.D., Departamento de Fisiología y Farmacología, Edificio Departamental, Campus Miguel de Unamuno, 37007 Salamanca, Spain.
E-mail: jmlnovoa@usal.es

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