Role of Intracellular Calcium Mobilization and Cell-Density-Dependent Signaling in Oxidative-Stress-Induced Cytotoxicity in HaCaT Keratinocytes

Edina Bakondi,*[1] Mónika Gönczi,†‡[1] Éva Szabó,§¶ Péter Bai,* Pál Pacher,¶ Pál Gergely,* László Kovács,†‡ János Hunyadi,§ Csaba Szabó,¶** László Csernoch,†‡ and László Virág*¶

*Departments of Medical Chemistry and †Physiology, Medical and Health Science Center, Research Center for Molecular Medicine, University of Debrecen, Hungary; ‡Cell Physiology Research Group of the Hungarian Academy of Sciences, Debrecen, Hungary; §Department of Dermatology, University of Debrecen, Debrecen, Hungary; ¶Inotek Pharmaceuticals Corporation, Beverly, Massachusetts, USA; **Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary

Peroxynitrite is a nitric-oxide-derived cytotoxic mediator produced in a broad range of inflammatory conditions, ranging from sunburn erythema to contact hypersensitivity. Our previous work has shown that in HaCaT cells the cytotoxic activity of peroxynitrite involves both apoptotic and necrotic routes with poly (ADP-ribose) polymerase activation serving as a molecular switch diverting the default apoptotic pathway toward necrosis. Nonetheless, keratinocytes are regarded as highly resistant toward environmental noxa including oxidative stress. We set out to investigate the possible role of two parameters, intracellular calcium mobilization and high cell density, in protecting HaCaT cells from peroxynitrite/oxidative-stress-induced cytotoxicity. First we characterized the effect of peroxynitrite on the calcium homeostasis of HaCaT cells and demonstrated that both authentic peroxynitrite and the peroxynitrite generating compound 3-morpholino-sydnonimine triggered an elevation in intracellular calcium levels. Moreover, we established that treatment of cells with the cell-permeable calcium chelator BAPTA-AM provided significant cytoprotection against peroxynitrite- and hydrogen-peroxide-induced cytotoxicity. Furthermore, when cells reached confluence they were highly resistant to the toxic effects of peroxynitrite, hydrogen peroxide, and superoxide. The resistance to oxidative stress provided by calcium chelation and high cell density involved inhibiting the activation of both poly(ADP-ribose) polymerase and caspases. Our data may provide an explanation for the resistance to oxidative stress of superficial, highly differentiated keratinocytes and indicate that basal proliferative keratinocytes are sensitive *in vivo* targets of oxidative stress injury. ***Key words: apoptosis/calcium signaling/hydrogen peroxide/necrosis/peroxynitrous acid. J Invest Dermatol 121:88–95, 2003***

Nitric oxide (NO) has recently emerged as an important mediator of various physiologic functions and pathophysiologic conditions in the skin (Weller, 1997; Virág *et al*, 2002). NO is produced by a family of enzymes called NO synthases through enzymatic oxidation of the guanidino group of L-arginine (Marletta *et al*, 1998). Moreover, in the skin, the nonenzymatic reduction of sweat nitrate can also give rise to NO production (Weller *et al*, 1996), which is thought to contribute to the barrier function of the skin. In addition, NO has been shown to regulate cutaneous blood flow, melanogenesis, and wound healing (Warren, 1994; Romero-Graillet *et al*, 1996; Yamasaki *et al*, 1998). Most cell types residing in the skin have also been reported to produce NO in response to appropriate stimulation (Weller, 1997; Virág *et al*, 2002). For example, keratinocytes, Langerhans cells, dermal fibroblasts, and melanoma cells express inducible NO synthase upon stimulation with inflammatory cytokines and/or lipopolysaccharide (Weller, 1997). Keratinocytes have also been shown to contain constitutive NO synthase that can be activated by ultraviolet-B exposure (Deliconstantinos *et al*, 1996).

Excess production of NO, especially when combined with increased oxyradical production, may lead to the formation of peroxynitrite (Beckman and Koppenol, 1996). Peroxynitrite ($ONOO^-$) is a powerful oxidant formed in the near diffusion-limited reaction between NO and superoxide. Peroxynitrite is the most likely final cytotoxic mediator of tissue injury observed in association with increased NO production, e.g., in inflammation, shock, or reperfusion injury (Szabó, 1996). An increasing body of evidence suggests that peroxynitrite can also be produced in the skin (Virág *et al*, 2002). Peroxynitrite was shown to be produced in sunburn erythema, burns, contact hypersensitivity, and systemic sclerosis (Hattori *et al*, 1996; Cotton *et al*, 1999; Rawlingson *et al*, 2000; Szabó *et al*, 2001; Virág *et al*, 2002). The cellular effects of peroxynitrite on keratinocytes and on other

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[1]The first two authors contributed equally to the work.

Reprint requests to: László Virág, Department of Medical Chemistry, University of Debrecen, Bem tér 18/B, H-4026 Debrecen, Hungary, Email: viraglaszlo@hotmail.com; or László Csernoch, Department of Physiology, University of Debrecen, H-4012 Debrecen, PO Box 22, Hungary, Email: csl@phys.dote.hu

Abbreviations: MTT, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide; PARP: poly(ADP-ribose) polymerase; SIN-1: 3-morpholino-sydnonimine.

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skin-derived cell types, however, have not been clearly defined. In our previous work we investigated the mechanisms of peroxynitrite-induced cytotoxicity in HaCaT cells (Szabó *et al*, 2001). We demonstrated that peroxynitrite activates poly(ADP-ribose) polymerase (PARP), a nuclear nick sensor enzyme, the inhibition of which protects cells from oxidative damage (Szabó *et al*, 2001). According to the "cellular suicide model" (Berger *et al*, 1983), overactivation of PARP consumes its substrate (NAD^+) and also depletes cellular ATP stores leading to cell death. Through this cellular energetic failure (NAD^+ and ATP depletion) PARP activation serves as a molecular switch mechanism diverting the "default" apoptotic cell death toward necrotic cell death (Virág *et al*, 1998a; 1998b; Ha and Snyder, 1999; Virág and Szabó, 2002). In HaCaT cells, as in other cell types, the cytoprotective effect of PARP inhibitors, evidenced by using both the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay (Szabó *et al*, 2001) and propidium iodide uptake (Bakondi and Virág, unpublished), was accompanied by an increased output of apoptotic DNA fragmentation.

The factors determining the sensitivity of keratinocytes to oxidative stress are unknown to date. Due to the barrier function of the skin, the keratinocytes that provide the first line of defense must be resistant to environmental noxa, including oxidative stimuli. Indeed, keratinocytes are generally believed to be resistant to oxidative stress. The factors affecting the oxidative stress sensitivity/resistance of these cells are not well understood, however. Moreover, epidermal keratinocytes do not represent a homogeneous population. We hypothesized that basal proliferative keratinocytes differ from superficial highly differentiated counterparts in terms of their sensitivity to oxidative stress.

We have identified two factors (calcium signaling and cell density) as potential determinants of sensitivity to oxidative stress in keratinocytes. Calcium signaling is considered to regulate various cell death processes (Nicotera and Orrenius, 1998), including peroxynitrite toxicity (Virág *et al*, 1999; Gutierrez-Martin *et al*, 2002). In addressing the epidermal calcium gradient in the skin (Yuspa *et al*, 1988) we initially examined the potential role of calcium signaling in regulating the sensitivity of keratinocytes to peroxynitrite. The second aim of our work was to establish whether or not proliferating and differentiated keratinocytes differ in their sensitivity to peroxynitrite. These studies were undertaken in HaCaT cells, which represent a suitable cellular model of proliferating keratinocytes. Furthermore, upon reaching confluence in culture HaCaT cells undergo differentiation accompanied by marked alterations in various cellular functions (Ryle *et al*, 1989). Finally, we went on to investigate the relationship between oxidative-stress-induced PARP activation, calcium mobilization, and cell density signaling in HaCaT cells.

MATERIALS AND METHODS

Cell culture HaCaT keratinocytes were cultured in an endotoxin-free modified Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and 10 μl per ml antibiotic, antimycotic solution (Sigma, Budapest, Hungary) as described previously (Csernoch *et al*, 2000). Calcium measurements were carried out on presheet cultures. Cells were washed twice with a buffer containing 137 mM NaCl, 5.4 mM KCl, 6.9 mM $NaHCO_3$, 0.5 mM Na_2 ethylenediamine tetraacetic acid, and 1 g per liter glucose, pH 7.4, and trypsinized at 37°C for 10 min. Serum supplemented DMEM was then added back to culture flasks to neutralize trypsin. Trypsinized keratinocytes were centrifuged at 1000*g* for 10 min and plated ($1–2 \times 10^5$ per ml) on sterile coverslips (for calcium measurements), on 96-well plates (cytotoxicity assay), or on 12-well plates (caspase assay, PARP activity assay). Cells were maintained in DMEM at 37°C in 5% CO_2 for further studies.

$[Ca^{2+}]_i$ measurement Changes in $[Ca^{2+}]_i$ were detected with Fura-2 as described in our previous reports (Bíró *et al*, 1998; Csernoch *et al*, 2000). Briefly, the calcium-sensitive probe was introduced into the intracellular space by incubating the keratinocytes in 5 μM Fura-2 AM for 1 h at 37°C. Before each measurement the cells were maintained at room temperature (22–24°C) in normal Tyrode's solution (in mM, 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 11.8 HEPES–NaOH, 1 g per liter glucose, pH 7.4) for a half an hour to allow homogeneous distribution of the dye.

The coverslips, containing the Fura-2 loaded cells, were then placed on the stage of an inverted fluorescence microscope (Diaphot, Nikon, Kowasaki, Japan). Excitation was altered between 340 and 380 nm using a dual wavelength monochromator (Deltascan, Photon Technology International, Lawrenceville, KY, USA). The emission was monitored at 510 nm with a photomultiplier at an acquisition rate of 10 Hz per ratio. $[Ca^{2+}]_i$ levels were calculated according to the method of Grynkiewicz *et al* (1985) from the ratio ($R = F_{340}/F_{380}$) of the fluorescence intensities measured with excitation wavelengths 340 nm (F_{340}) and 380 nm (F_{380}) as previously described (Bíró *et al*, 1998; Csernoch *et al*, 2000) using *in vivo* calibration data.

Chemicals All experiments measuring $[Ca^{2+}]_i$ were performed in either normal Tyrode's solution or a calcium-free Tyrode's solution of the same composition, with 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid and no added $CaCl_2$. Peroxynitrite was dissolved in modified (HEPES- and glucose-free) Tyrode's solution (pH 11) immediately before use. (Alkaline pH prevents decomposition of the oxidant.) 3-Morpholino-sydnonimine (SIN-1) was also dissolved in modified Tyrode's solution, except that the pH of this solution was set to 7.5. The vehicle solutions and decomposed peroxynitrite were also tested to determine if they, by themselves, were capable of altering $[Ca^{2+}]_i$. Fura-2 AM was purchased from Molecular Probes (Eugene, OR); all other chemicals were from Sigma.

Agonists were applied through a fast perfusion system that allowed a rapid and local application of the compound onto the investigated cell (Bíró *et al*, 1998). Note that this arrangement ensured a constant peroxynitrite concentration around the studied cell during perfusion.

Cytotoxicity assays Cell viability was assessed by the colorimetric MTT assay, as described previously (Szabó *et al*, 2001). Briefly, cells were pretreated with BAPTA-AM (0–2.5 μM), glutathione (5 mM), N-acetyl cysteine (5 mM), or superoxide dismutase (2.5 kU per ml) + catalase (4 kU per ml) for 30 min followed by treatment with either authentic peroxynitrite, SIN-1, hydrogen peroxide, or the superoxide-generating system (500 μM xanthine + 10 U per ml xanthine oxidase). The intensity of oxidative stimuli was adjusted so that the degree of cytotoxicity was in the range 60%–80%. (At low cytotoxicity values, mechanistic investigations are problematic, whereas at cytotoxicity higher than 90% cytoprotection is often difficult to achieve.) Four hours later MTT was added to the cells (0.5 mg per ml) and incubated for an additional hour. The medium was then aspirated and the formazan crystals were dissolved by the addition of 100 μl dimethylsulfoxide. Optical density was determined in a Multiskan MS plate reader (Labsystem, Vantaa, Finland) at 550 nm test wavelength with 690 nm as a reference wavelength.

Measurement of caspase activation Caspase activity was measured with the Caspase-3 Intracellular Activity Assay kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. The assay is based on the use of the fluorogenic peptide substrate PhiPhiLux. The septapeptide containing the DEVD cleavage site of caspase-3-like enzymes is conjugated to two fluorochromes, the fluorescence of which is quenched in the uncleaved folded peptide. Upon cleavage of the substrate the fluorophores provide an intense green fluorescence signal. Six hours after peroxynitrite treatment, cells were scraped into medium and pelleted by centrifugation. Cells were then incubated with the substrate for 60 min at 37°C and analyzed by flow cytometry.

PARP activity assay PARP activity was measured as previously described (Szabó *et al*, 2001). The medium was removed from the cells 20 min after peroxynitrite treatment and cells were incubated at 37°C in 0.5 ml assay buffer (56 mM HEPES pH 7.5, 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, and 0.125 μM ^{3}H-NAD (0.5 μCi per ml)). Cells were then scraped and transferred into Eppendorf tubes. 200 μl ice-cold 50% trichloroacetic acid was added to the samples and tubes were incubated for 4 h at 4°C. Samples were then centrifuged for 10 min at 10,000*g*, and the pellets were washed twice in ice-cold 5% trichloroacetic acid and solubilized overnight in 250 μl 2% sodium dodecyl sulfate/0.1 N NaOH at 37°C. The contents of the tubes were added to 7 ml ScintiSafe Plus scintillation liquid (Fisher Scientific) and radioactivity was determined in a liquid scintillation counter (Wallac, Gaithersburg, MD).

Statistical analysis All values are expressed as either mean ± SEM (calcium levels in **Figs 3** and **4**) or mean ± SD of quadruplicate samples

(all other data) and experiments were repeated at least three to four times. Data sets were analyzed by either Student's paired *t* test (calcium levels in **Figs 3** and **4**) or one-way analysis of variance (ANOVA) followed by Tukey's *posthoc* test (all other data). A significance level of $p < 0.05$ was considered significant.

RESULTS

SIN-1 alters $[Ca^{2+}]_i$ by releasing calcium from the intracellular stores and by inducing an influx from the extracellular environment To test if the application of the peroxynitrite generating agent SIN-1 would alter $[Ca^{2+}]_i$, and also to assess the source of calcium ions entering the cytoplasm, SIN-1 (1, 2.5, and 5 mM) was applied to the cells in the presence and absence of extracellular calcium. To monitor the loading of the internal calcium stores the well-known phosphoinositide activator ATP (180 μM) was administered before and after the application of SIN-1 (**Fig 1**).

With external calcium present SIN-1 (1 mM) caused a marked increase in $[Ca^{2+}]_i$. The amplitude of the ATP-evoked calcium transient subsequent to SIN-1 application, however, was not significantly reduced compared to the transient obtained prior to the application of SIN-1 (**Fig 1**). Although the elevation in $[Ca^{2+}]_i$ during SIN-1 application was greater when higher concentrations of the drug were administered, the alteration in the amplitude of the ATP-evoked calcium signal was essentially the same (compare **Fig 1*A, B***).

When calcium was removed from the extracellular solution (**Fig 2**) SIN-1 was still capable of causing an increase in $[Ca^{2+}]_i$. This increase, although much less pronounced than that shown in **Fig 1**, demonstrates that the effect of SIN-1, at least in part, is due to the mobilization of calcium from internal stores. In contrast to the data in **Fig 1**, in the absence of external calcium the ATP-evoked calcium transient following SIN-1 application was markedly reduced in amplitude compared to that obtained before the application of SIN-1. There also appeared to be a concentration-dependent response, with higher SIN-1 concentration causing a greater reduction in the amplitude of the ATP-evoked calcium signal.

The results shown in **Fig 3** were averaged from several experiments where more than 60 individual cells were studied. As demonstrated in **Fig 3(*A*)**, SIN-1 caused a concentration-

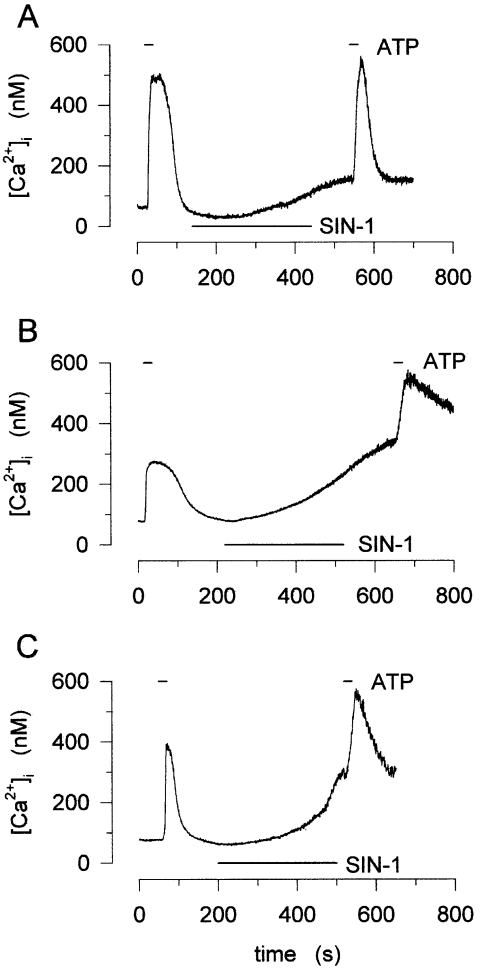

Figure 1. Changes in intracellular calcium concentration in response to the application of ATP and SIN-1 in the presence of external calcium (1.8 mM). ATP (180 μM) was applied for 20 s and SIN-1 for 300 s, as indicated by the horizontal lines. The effect of 1 mM (*A*), 2.5 mM (*B*), and 5 mM SIN-1 (*C*) is shown. Note that SIN-1 caused a gradual increase in $[Ca^{2+}]_i$. Data presented are from three different cells, representative of six, eight, and eight independent experiments carried out for (*A*), (*B*), and (*C*), respectively.

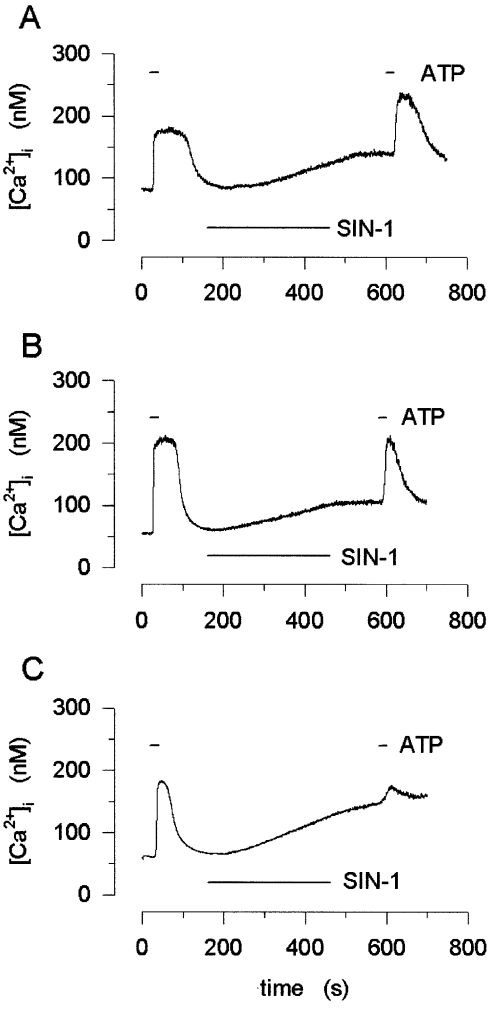

Figure 2. Changes in intracellular calcium concentration in response to the application of ATP and SIN-1 in the absence of external calcium (1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid). ATP (180 μM) was applied for 20 s and SIN-1 for 300 s, as indicated by the horizontal lines. The effect of 1.0 mM (*A*), 2.5 mM (*B*), and 5 mM SIN-1 (*C*) is shown as in **Fig 1**. Note that SIN-1 not only caused a gradual increase in $[Ca^{2+}]_i$ but substantially reduced the amplitude of the second ATP-evoked calcium signal. Note also the altered *y*-axis scale compared to **Fig 1**. Data presented are from three different cells, representative of 11, 14, and 15 independent experiments carried out for (*A*), (*B*), and (*C*), respectively.

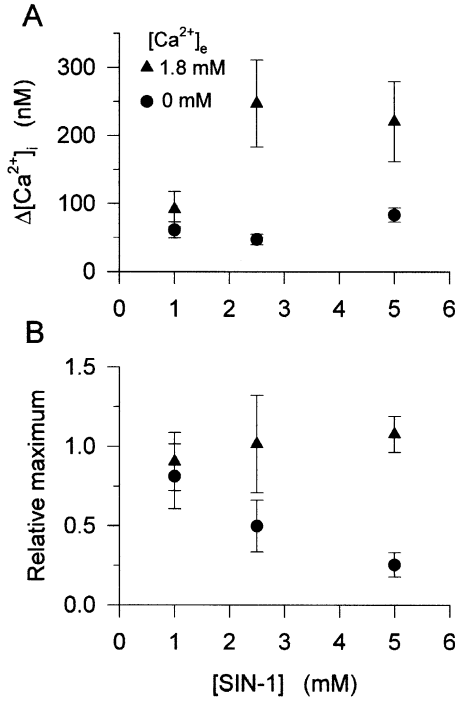

Figure 3. Concentration-dependent effects of SIN-1 on $[Ca^{2+}]_i$ and on the ATP-evoked calcium signals. Measurements were carried out in the presence (1.8 mM in Tyrode's solution; *squares*) and absence (*circles*) of external calcium ($[Ca^{2+}]_e$). Values shown on both *panel A* and *panel B* represent mean ± SEM with *n* in the range 6–15. *Panel A* shows the increase in $[Ca^{2+}]_i$ caused by 300 s application of SIN-1 at the given concentration. At all SIN-1 concentrations tested, statistically significant calcium mobilization was found both in the presence of 1.8 mM extracellular calcium (at 1 mM SIN $p<0.05$, at 2.5 mM SIN $p<0.03$, at 5 mM SIN $p<0.03$) and in the absence of extracellular calcium (at 1 mM SIN $p<0.01$, at 2.5 mM SIN $p<0.01$, at 5 mM SIN $p<0.01$) compared to baseline values (Student's paired *t* test). *Panel B* shows the effect of SIN-1 on the amplitude of the ATP-evoked increase in $[Ca^{2+}]_i$ measured subsequent to SIN-1 application. With extracellular calcium present, SIN-1 had no significant effect on the ATP-evoked calcium signal (at 1 mM $p>0.5$, at 2.5 mM SIN $p>0.4$, at 5 mM SIN $p>0.8$). In the absence of extracellular calcium, treatment of cells with 2.5 or 5 mM SIN-1 resulted in a significantly reduced ATP-evoked calcium signal (at 1 mM $p>0.4$, at 2.5 mM SIN $p<0.05$, at 5 mM SIN $p<0.02$).

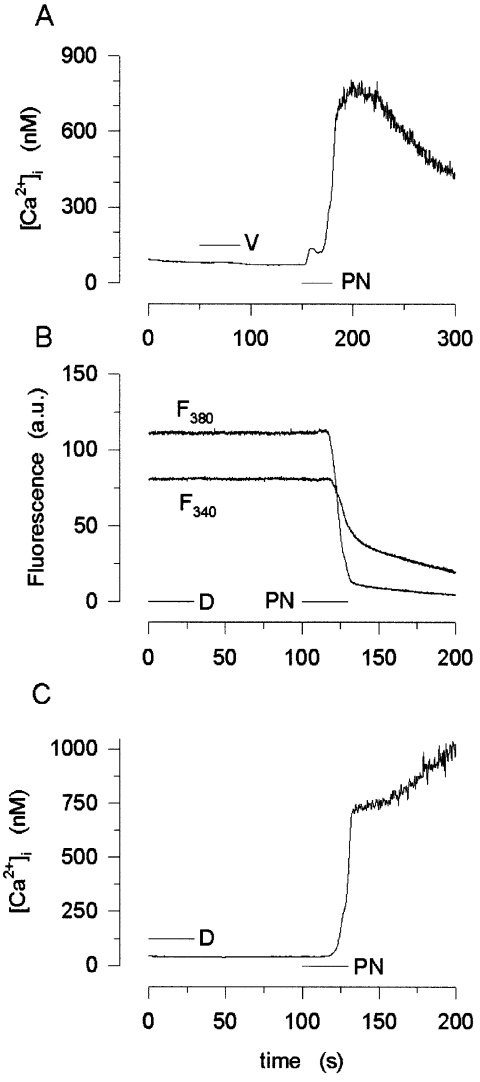

Figure 4. The effect of peroxynitrite (PN, 10 μM), decomposed PN (D), or the vehicle (V, see *Materials and Methods*) on $[Ca^{2+}]_i$. (*A*) The vehicle and peroxynitrite were applied subsequently (for 40 and 30 s, respectively) as indicated. Note that whereas the vehicle had no measurable effect peroxynitrite caused a substantial increase in $[Ca^{2+}]_i$. (*B*) Peroxynitrite caused a non-specific increase in membrane permeability indicated here as a drop in fluorescence (arbitrary units, a.u.), both at 340 and 380 nm. (*C*) Changes in $[Ca^{2+}]_i$ calculated from the fluorescence signals shown in *panel B*. Note the increase in noise (in *A* and *C*) that is attributed to the low fluorescence levels in these cells after the application of peroxynitrite. Data are presented from two different cells, representative of 13 experiments. Statistical analysis (Student's paired *t* test) showed that neither vehicle nor decomposed peroxynitrite caused significant elevation in intracellular calcium ($p>0.08$ for vehicle, $p>0.11$ for decomposed peroxynitrite). Peroxynitrite-induced elevation in intracellular calcium was statistically significant ($p<0.04$). (Note that in some peroxynitrite-treated cells, calcium level reached micromolar range where saturation of the dye makes the measurement less accurate. Omission of these cells from the statistical evaluation results in a higher degree of significance: $p<0.003$.)

dependent elevation in $[Ca^{2+}]_i$, reaching approximately 220 nM at the highest concentration (5 mM) used when calcium was present in the extracellular solution. Although the increase in $[Ca^{2+}]_i$ in the absence of external calcium was not as marked, only reaching 80 nM with 5 mM SIN-1, this increase was still statistically significant. By comparison, no noticeable decrease in the amplitude of the ATP-evoked response was detected when calcium was present in the extracellular solution. When calcium was removed from the external environment the amplitude of the ATP-evoked calcium transient was concentration-dependently reduced. These data clearly indicate that SIN-1 was capable of mobilizing calcium from internal stores and inducing the influx of calcium through the surface membrane. It should be noted that, when calcium influx was enabled, SIN-1 did not significantly lower the content of the intracellular stores.

Peroxynitrite opens an influx pathway for calcium The application of 10 μM peroxynitrite resulted in a dramatic increase in $[Ca^{2+}]_i$ that was not usually reversible, or only slightly so, after its removal, as demonstrated in **Fig 4(*A*), (*C*)**. Interestingly, the relative noise in the calcium signals increased beyond the level that was expected from the saturation of the dye. Examining the absolute fluorescence (**Fig 4*B***) values revealed a decrease in total cell fluorescence both at 340 and 380 nm, indicating a loss of dye from the intracellular solution. These findings point to a membrane-perturbing effect of peroxynitrite causing both an influx of calcium and a partial leakage of the calcium-sensitive probe. The observations that $[Ca^{2+}]_i$ began to

decline following the removal of peroxynitrite and that the measured fluorescence ratio (3.8 at the maximum for the trace in **Fig 4*A***) was far from R_{max} (8.6 from calibration data, Bíró *et al*, 1998) indicate that the integrity of the cell membrane was not completely lost during the treatment.

To exclude the possibility that the vehicle solution (pH = 11) or peroxynitrite decomposition products were responsible for the above described effects, in the experiments presented in **Fig 4** either the vehicle (**Fig 4*A***) or decomposed peroxynitrite (**Fig 4*B, C***) were also applied. These treatments did not cause any measurable elevation in $[Ca^{2+}]_i$. The subsequent addition of peroxynitrite, however, resulted in the already mentioned dramatic increase in $[Ca^{2+}]_i$. These observations demonstrate the ability of peroxynitrite to induce an influx of calcium into HaCaT keratinocytes, presumably through a non-specific pathway.

Buffering of intracellular calcium by BAPTA-AM protects HaCaT cells from peroxynitrite-induced cytotoxicity As our previous work has shown that peroxynitrite is cytotoxic to HaCaT cells (Szabó *et al*, 2001) we went on to investigate whether or not peroxynitrite-induced calcium mobilization contributes to the cytotoxic effects of the oxidant. In this study HaCaT cells were pretreated with the cell-permeable calcium chelator BAPTA-AM for 30 min and then exposed to peroxynitrite. BAPTA-AM provided significant protection from peroxynitrite-induced cytotoxicity (**Fig 5*A***). Moreover, cytotoxicity induced by the peroxynitrite-releasing compound SIN-1 was also reduced by intracellular calcium chelation, although to a lesser extent (**Fig 5*A***). In order to establish whether this phenomenon is peroxynitrite specific or, as expected, is a general feature of oxidative stress, we used hydrogen peroxide and superoxide as triggers of oxidative stress in HaCaT cells. Whereas BAPTA-AM exerted significant cytoprotective effects in hydrogen-peroxide-treated cells (**Fig 5*B***), it provided no significant protection against superoxide (**Fig 5*C***). In order to show that superoxide-induced HaCaT cell death is amenable to pharmacologic intervention, we pretreated the cells with 5 mM glutathione, 5 mM N-acetyl cysteine, and 80 U per ml superoxide dismutase + 125 U per ml catalase. These antioxidants provided 72.7% ± 4.4%, 55.0% ± 5.6%, and 94.5% ± 9% protection against superoxide-induced cytotoxicity, respectively.

Cell-density-dependent signals modulate peroxynitrite sensitivity of HaCaT cells Upon reaching confluence HaCaT cells undergo differentiation (Ryle, 1989), a process similar to that occurring in the skin during passage of keratinocytes from the basal towards the superficial layers of the epidermis. We examined the effect of this cell-contact-induced differentiation process on the sensitivity of HaCaT cells to peroxynitrite. HaCaT keratinocytes were seeded in 96-well tissue culture plates at different densities and were allowed to adhere overnight. On the following day, cultures with approximate densities of 20%, 40%, 60%, 80%, and 100% were exposed to peroxynitrite, hydrogen peroxide, or superoxide (xanthine/xanthine oxidase). After 4 h, cell viability was determined by the MTT assay. Our data showed that subconfluent cultures of HaCaT cells (20%–80%) displayed equal sensitivity to oxidative stress (**Fig 6**). In contrast, confluent cultures demonstrated significant resistance to peroxynitrite, hydrogen peroxide, and superoxide (**Fig 6**). The resistance of confluent cultures to oxidative stress could result from either the production of a soluble mediator by the cells or direct cell-to-cell contact. To determine which of these may be the case, cytotoxicity assays were repeated in confluent cells where the culture medium was replaced just prior to oxidant treatment. Changing the medium, however, did not alter the resistance of confluent cultures (data not shown) indicating that cell-to-cell contact and not a soluble mediator was responsible for this phenomenon.

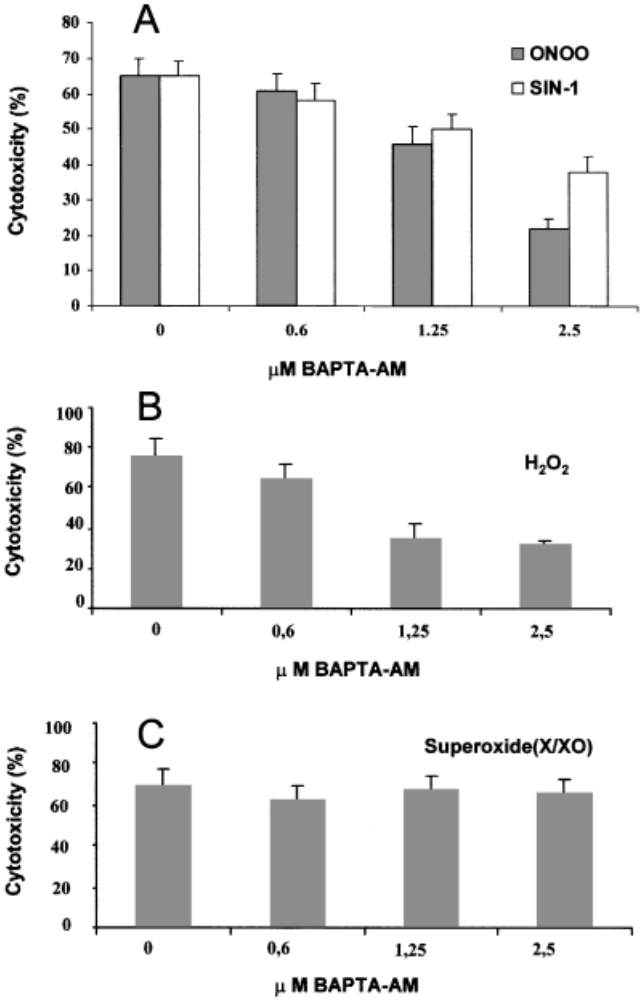

Figure 5. Role of calcium mobilization in the oxidative-stress-induced cytotoxicity of HaCaT cells. HaCaT cells were seeded at 80% density in 96-well plates and were treated with different concentrations (0–2.5 μM) of BAPTA-AM for 30 min. Cells were then treated with 125 μM authentic peroxynitrite (*A*), 2.5 mM SIN-1 (*A*), 250 μM hydrogen peroxide (*B*), or the superoxide generating system 500 μM xanthine + 10 U per ml xanthine oxidase (*C*). After 4 h, cytotoxicity was determined with the MTT assay. Results are presented as mean ± SD of quadruplicate samples. The experiment was repeated three times.

BAPTA-AM and high density signaling inhibits oxidant-induced PARP activation in HaCaT cells Our previous work has identified PARP activation as a mechanism responsible for peroxynitrite-induced HaCaT cell death. Here we set out to investigate whether the protective effects of intracellular calcium buffering and high cell density are related to PARP activation. Indeed we have found that BAPTA-AM significantly inhibited peroxynitrite-induced PARP activation in HaCaT cells (**Fig 7**). Similarly, high density HaCaT cultures were resistant to peroxynitrite-induced PARP activation (**Fig 7**). The effects of BAPTA-AM and high density signaling on PARP activation were not additive, however.

Effect of BAPTA-AM and cell density signaling on peroxynitrite-induced caspase activation in HaCaT cells Peroxynitrite-induced cytotoxicity is characterized by both apoptotic and necrotic features (Virág *et al*, 1998a; 1998b; Szabó *et al*, 2001). We, and others, have previously shown that in various cell lines (e.g., HaCaT, thymocytes, fibroblasts) the switch from the default apoptotic cell death toward necrotic

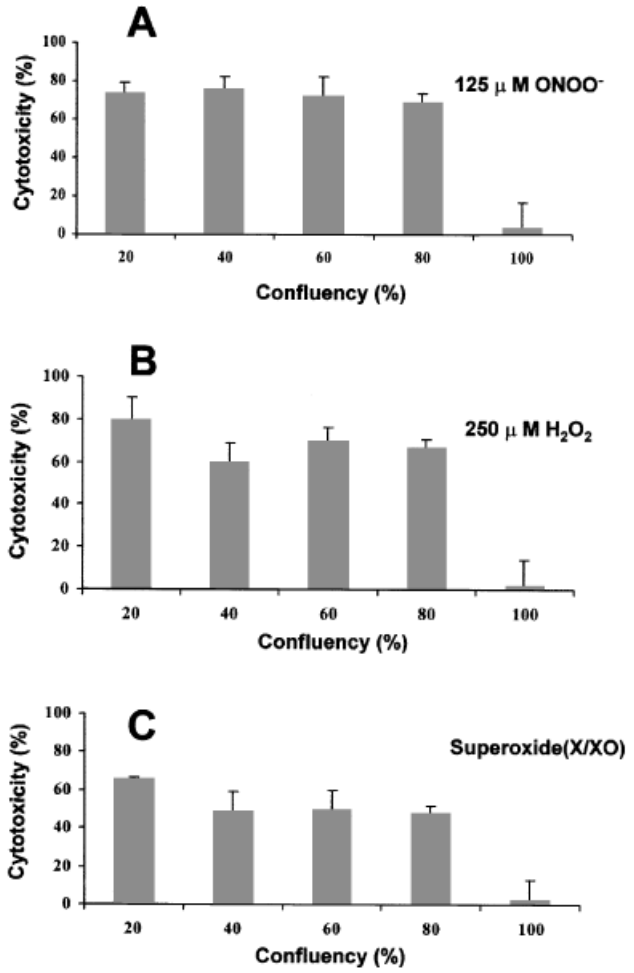

Figure 6. Role of cell density in the oxidative-stress-induced cytotoxicity of HaCaT cells. Cells were seeded in 96-well plates at different densities and were treated with 125 μM peroxynitrite (*A*), 250 μM hydrogen peroxide (*B*), or the superoxide generating system 500 μM xanthine + 10 U per ml xanthine oxidase (*C*). After 4 h, cytotoxicity was determined with the MTT assay. Data are presented as mean ± SD of quadruplicate samples. The experiment was repeated four times.

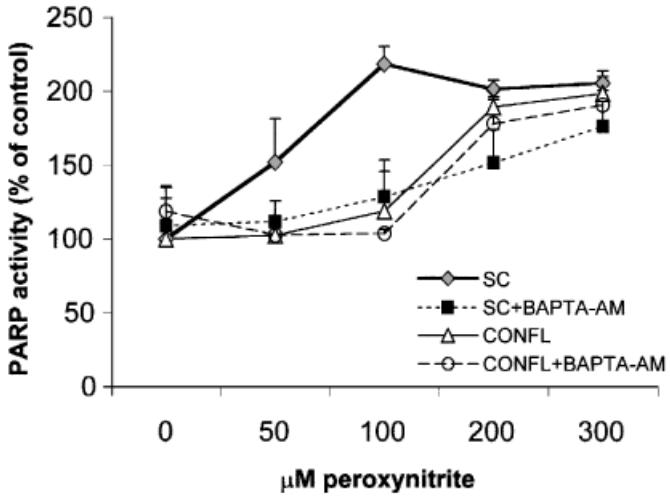

Figure 7. Effect of BAPTA-AM and cell density on peroxynitrite-induced PARP activation. Subconfluent (SC 80%) and confluent (CONFL 100%) HaCaT cells were treated with peroxynitrite in the presence or absence of BAPTA-AM (2.5 μM). After 30 min medium was replaced with permeabilization buffer containing ^{3}H-NAD. PARP activity was determined as ^{3}H incorporation into trichloroacetic acid precipitable proteins. Results are presented as mean ± SD of triplicate samples. The experiment was repeated three times.

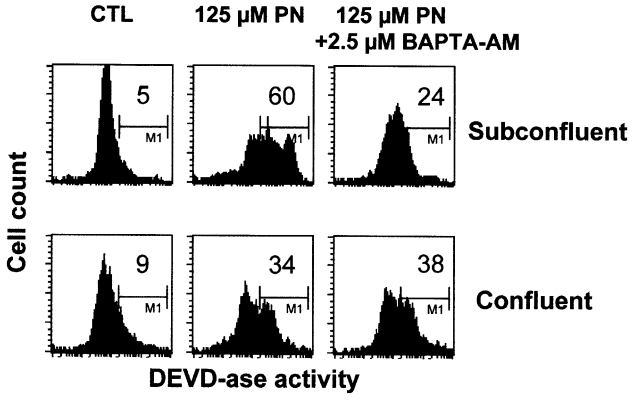

Figure 8. Effect of BAPTA-AM and cell density on peroxynitrite-induced caspase activation. Subconfluent (80%) and confluent (100%) HaCaT cells were treated with peroxynitrite in the presence or absence of BAPTA-AM (2.5 μM). After 6 h cells were stained with the fluorogenic PhiPhiLux caspase-3 substrate and analyzed by flow cytometry. Caspase activation is indicated by increased fluorescence (right shift). Numbers indicate the percentage of cells displaying increased fluorescence (caspase activation).

death is mediated, in part, by PARP activation as the enzyme depletes NAD^+ and ATP pools and thereby compromises the energy-dependent apoptotic pathway (Virág *et al*, 1998a; 1998b; Filipovic *et al*, 1999; Ha and Snyder, 1999). In this study we investigated the effects of calcium mobilization and cell density on peroxynitrite-induced caspase activation, a biochemical parameter of apoptotic cell death. Peroxynitrite induced caspase-3-like activity in HaCaT cells, with 60% of the cells displaying higher DEVD-ase activity compared to only 5% of the cells in the control samples (**Fig 8**). Pretreatment of the cells with BAPTA-AM significantly inhibited peroxynitrite-induced caspase activity (**Fig 8**). In confluent cultures, caspase activation was less notable and BAPTA-AM treatment could not further reduce caspase activation (**Fig 8**).

DISCUSSION

The primary aim of our work was to establish whether or not calcium signals promote cell death in peroxynitrite-treated HaCaT cells. Both peroxynitrite and the peroxynitrite-generating compound SIN-1 induced elevations in intracellular calcium concentrations in HaCaT cells. This finding is in line with previous observations reporting that peroxynitrite induced calcium mobilization in thymocytes, neurons, and isolated mitochondria (Packer and Murphy, 1994; Virág *et al*, 1999; Ohkuma *et al*, 2001). Whereas the source of intracellular calcium "signal" was mainly the extracellular compartment, the release of calcium from intracellular sources was also significant. Influx of calcium from the extracellular compartment may be due to a direct damaging effect of peroxynitrite on the plasma membrane or may indicate opening of store-operated calcium channels. Notably, HaCaT cells have previously been shown to have such channels (Csernoch *et al*, 2000).

Buffering intracellular calcium by the cell-permeable calcium chelator BAPTA-AM provided significant protection from peroxynitrite toxicity, indicating the involvement of a calcium signal in the cell death process. The cytoprotective effect of BAPTA-AM

was not restricted to peroxynitrite-treated cells, however. The chelator also inhibited hydrogen-peroxide-induced cytotoxicity suggesting a general role for a calcium signal in mediating the cytotoxic effects of oxidative stress. The cytoprotective effect of intracellular calcium chelation is in accordance with previous observations reporting beneficial effects of calcium chelators in oxidatively stressed renal tubular cells (Ueda and Shah, 1992), glutamate-treated cortical neurons (Frandsen and Schousboe, 1991), and excitotoxic and ischemic neuronal injury (Tymianski *et al*, 1993). Interestingly, in our study calcium chelation did not protect HaCaT cells from the cytotoxic effect of superoxide indicating that distinct cytotoxic pathways are triggered by different reactive species.

Our previous work identified the existence of multiple cell death pathways in the peroxynitrite-induced cytotoxicity in HaCaT cells (Szabó *et al*, 2001). At lower concentrations of the oxidant, caspase-mediated apoptotic cell death predominates. At more intense oxidative stress, however, the apoptotic machinery is disabled and HaCaT cells die through a PARP-mediated necrotic mechanism (Szabó *et al*, 2001). The "apoptosis to necrosis switch role" of PARP probably involves depletion of intracellular stores of NAD^+ and ATP, thus interfering with energy-dependent steps of the apoptotic machinery (Virág *et al*, 1998a; Ha and Snyder, 1999; Virág and Szabó, 2002). In our work, chelation of intracellular calcium by BAPTA-AM inhibited peroxynitrite-induced PARP activation. Similar findings have previously been reported in thymocytes, Caco-2 colon carcinoma cells, and neonatal rat brain slices (Tasker *et al*, 1998; Karczewski *et al*, 1999; Virág *et al*, 1999). As PARP-1 does not require calcium for activity (Virág *et al*, 1999), calcium signaling is likely to be a proximal step of a signal transduction pathway leading to PARP activation. In U937 cells Cantoni's laboratory found that exposure of cells to tert-butyl hydroperoxide or peroxynitrite led to calcium-dependent overproduction of reactive oxygen species in mitochondria (Guidarelli *et al*, 1997; 2000). Furthermore, secondary radicals rather than the primary oxidative stimuli were found to be responsible for DNA strand scission (Guidarelli *et al*, 1997; 2000). Taken together, these data suggest that a calcium signal is required for mitochondrial perturbations leading to reactive oxygen intermediate overproduction, DNA breakage, and eventually PARP activation. Moreover, calcium chelation has been found to inhibit DNA breakage in oxidatively stressed thymocytes (Virág *et al*, 1999). In addition, an inositol 1,4,5,-trisphosphate mediated, calcium signal-dependent PARP activation process has recently been described in neuronal cells (Homburg *et al*, 2000), providing support for our hypothesis. The exact mechanism by which an elevation of the intracellular calcium level evokes DNA breakage and PARP activation has yet to be defined, however.

Based on the PARP inhibitory effect of calcium chelation, and given the "apoptosis to necrosis switch" role of PARP, we would expect BAPTA-AM-treated HaCaT cells to be diverted toward apoptosis. In contrast, BAPTA-AM significantly reduced peroxynitrite-induced caspase activation, indicating that calcium is likely to be involved in the peroxynitrite-induced apoptotic process in HaCaT cells. As caspase activity does not directly depend on calcium (Stennicke and Salvesen, 1997), elevated intracellular calcium levels may be involved in signaling peroxynitrite-induced apoptosis at a step proximal to caspase activation.

The other major finding of our work is that the sensitivity of HaCaT cells to oxidative stress is dependent on the cell density. In light of our data, the marked oxidative stress resistance of confluent cultures may be explained by reduced PARP activation and by inhibition of caspase-mediated apoptotic pathways. We hypothesize that different metabolic activity (e.g., mitochondrial) may be responsible for the variation in sensitivity to oxidative stress observed between subconfluent (proliferating) and confluent (differentiating) keratinocytes. The metabolic control of oxidative stress sensitivity is exemplified by the cytoprotective effect of Krebs' cycle substrates (Lee *et al*, 2001; Ying *et al*, 2002) and by the sensitizing effect of pentose phosphate pathway inhibition (Le Goffe *et al*, 2002).

The question arises whether calcium signaling and cell-density-evoked signaling are interrelated in regulating the sensitivity of keratinocytes to peroxynitrite. In the epidermis, superficial highly differentiated keratinocytes are resistant to oxidative stress (Vessey *et al*, 1995), which is in line with our *in vitro* data. Superficial keratinocytes are characterized by high intracellular calcium levels, however, compared to basal cells (Yuspa *et al*, 1988). Although these findings appear to contradict our data, this can be readily resolved as we propose that it is not the basal intracellular calcium level but changes in the intracellular calcium level that may serve as a cytotoxic signal. If $[Ca^{2+}]_i$ is elevated, peroxynitrite cannot induce a high enough cytotoxic signal, whereas in proliferating basal keratinocytes low basal $[Ca^{2+}]_i$ permits a high amplitude change in $[Ca^{2+}]_i$ upon treatment with peroxynitrite. Peroxynitrite-induced calcium signaling may, in turn, trigger DNA breakage and PARP activation. This scenario is supported by our previous findings that PARP activation could be observed only in the basal layer of the epidermis in sulfur-mustard-treated mouse skin (Virág *et al*, 2002). Moreover, our data suggest that under certain circumstances, e.g., during wound healing when cell contacts are disrupted and keratinocytes "dedifferentiate" into a proliferating phenotype, cells may be more vulnerable to oxidative stress than normal. Further studies are required to reveal the exact mechanism of peroxynitrite-induced calcium mobilization and to identify the cell-density-induced alterations in cellular metabolism that may be responsible for differential peroxynitrite sensitivity of proliferating and confluent cultures.

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