Calcium-dependence of hydrogen peroxide-induced c-fos expression and growth stimulation of multicellular prostate tumor spheroids

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Abstract **Hydrogen peroxide (H_2O_2) in nanomolar concentrations (20–100 nM) stimulated the growth of small (diameter 100 ± 30 μm) multicellular prostate cancer spheroids and increased c-fos expression. H_2O_2 transiently raised $[Ca^{2+}]_i$ by Ca^{2+} release from intracellular stores as the transient persisted in low (10 nM) Ca^{2+} solution but was abolished when intracellular Ca^{2+} stores were depleted by thapsigargin or chelation of $[Ca^{2+}]_i$ with BAPTA. The H_2O_2-induced $[Ca^{2+}]_i$ transient was furthermore inhibited by the P_2-purinoreceptor antagonists suramin and basilen blue, indicating that H_2O_2 may act via purinergic receptor stimulation. Treatment of spheroids with either suramin, basilen blue or BAPTA inhibited the H_2O_2-induced growth stimulation and c-fos expression, indicating that the H_2O_2-mediated growth stimulation of multicellular spheroids is mediated via a Ca^{2+}-dependent pathway.**

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Key words: Hydrogen peroxide; Calcium; P_2-purinoreceptor; c-fos; Tumor growth

1. Introduction

In recent years a growing body of evidence has emerged suggesting that low concentrations of reactive oxygen species (ROS) i.e. superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) play a crucial role in the stimulation of proliferative responses in a variety of preparations [1]. However, the mechanism(s) by which ROS stimulate cell cycle activity and proliferation is/are still controversial. It has been known for a long time that cancer cells endogenously produce ROS giving rise to the speculation that these ROS may be involved in cancer cell proliferation [2]. Additionally, ROS can be generated and released in response to specific cytokine/growth factor stimulation in a wide variety of normal and malignant cell types [3–5]. ROS have been shown to utilize the same signaling pathways as mitogens but also seem to directly turn on growth-related genes including c-fos, c-jun, c-myc, etc. [6–8]. Several nuclear transcription factors have been shown to be modulated by the redox-potential i.e. the DNA binding of the fos/jun heterodimer to the AP-1 promoter and the activation of nuclear factor kappa B (NFκB) by release from its inactive cytoplasmic complex with the inhibitory subunit IκB [9,10].

The involvement of H_2O_2 in mitogen-activated signal transduction pathways has been shown in experiments where exogenously added H_2O_2 stimulated tyrosine phosphorylation by the epidermal growth factor receptor [11,12] and activated the extracellular signal-regulated protein kinase (ERK2) which is a prominent component of the mitogen-activated protein kinase (MAPK) family [13]. ERK activation culminates in the phosphorylation of cytosolic and nuclear factors i.e. early immediate response genes which control a variety of cellular processes. A recent publication shows $O_2^{\cdot-}$, possibly produced by an NADPH-oxidase similar to the enzyme present in neutrophils, to be involved in ras-mediated signal transduction pathways leading to cell growth stimulation [14].

Signal transduction of most mitogens is transduced via Ca^{2+} signals and the pivotal role of Ca^{2+} in cell cycle activation is well established [15]. Interestingly the expression of growth-related genes such as c-fos, c-jun and nuclear transcription factors like NfκB has been shown to be as dependent on ROS as on Ca^{2+} [16,17]. As it has been demonstrated that H_2O_2 elicits intracellular $[Ca^{2+}]_i$ signals [17,18] an interdependence of $[Ca^{2+}]_i$-mediated signal transduction pathways and the action of ROS on cell proliferation are likely.

In the present study we show that nanomolar concentrations of H_2O_2 stimulate the growth of prostate multicellular tumor spheroids and transiently increase c-fos expression. Our data indicate that H_2O_2 activates growth factor receptors, presumably the P_2-purinoreceptor in the plasma membrane, and elicits a transient of $[Ca^{2+}]_i$ via release from intracellular stores. The observed growth stimulation by H_2O_2 is Ca^{2+}-dependent as inhibition of the H_2O_2-induced $[Ca^{2+}]_i$ transient abolished both, c-fos expression and stimulation of tumor growth.

2. Materials and methods

2.1. Chemicals

Basilen blue E-3G was from Sigma, Deisenhofen, Germany. Fluo-3, AM, BAPTA, AM, Pluronic F-127 and thapsigargin were from Molecular Probes, Eugene, USA. Suramin was obtained from RBI, Cologne, Germany.

2.2. Culture technique of multicellular spheroids

The human prostate cancer cell line DU-145 was kindly provided by Dr. J. Carlsson, Uppsala, Sweden. The cell line was grown routinely in 5% CO_2/humidified air at 37°C with Ham's F10 medium (Gibco, Live Technologies, Inc., Helgerman Court, MD, USA) supplemented with 10% fetal calf serum (Boehringer, Mannheim, Germany), 2 mM glutamine, 0.1 mM β-mercaptoethanol, 2 mM MEM, 100 IU/ml penicillin and 100 μg/ml streptomycin (ICN Flow, Meckenheim, Germany). Spheroids were grown from single cells. Cell monolayers were trypsinized with 0.2% trypsin, 0.05% EDTA (ICN Flow, Meckenheim, Germany) and seeded in siliconated 250 ml spin-

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Abbreviations: ROS, reactive oxygen species; BAPTA, 1,2-*bis*(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; NFκB, nuclear factor κB; ERK2, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; EDTA, ethyleneglycoltetraacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]

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ner flasks (Tecnomara, Fernwald, Germany) with 250 ml complete medium and agitated at 40 rpm using a Teche stirrer system (MCS-104S, Teche Ltd., Cambridge, UK). Cell culture medium was partially changed every day.

2.3. Incubation of spheroids with H_2O_2

Small multicellular spheroids (diameter 100 ± 30 μm) were washed in a modified F10 cell culture medium from which glutamine and β-mercaptoethanol were omitted. They were placed in ∅ 60 mm plastic nonadhesive culture dishes (Greiner, Solingen, Germany) and incubated for 1 h in modified F10 medium containing 20–100 nM H_2O_2. Spheroids were subsequently washed 3 times and cultured for 4 days in liquid overlay culture. Spheroid diameters in control and treated samples were monitored each day and spheroid volumes were calculated according to $V = 4/3 \cdot \pi \cdot r^3$. The life time of H_2O_2 in modified F10 cell culture medium and E1 buffer were determined by a luminol-dependent chemiluminescence assay [19]. The half life time for H_2O_2 in modified F10 medium was approximately 10 min. In E1 buffer no H_2O_2 degradation within 1 h of incubation was observed (data not shown).

2.4. Colony forming assay

H_2O_2-treated and control spheroids (diameter 100 ± 30 μm) were enzymatically dissociated with 0.2% trypsin, 0.05% EDTA (incubation time 5 min, 37°C), triturated and the enzymatic reaction was stopped by addition of 4 ml F10 medium to 1 ml trypsin/EDTA solution. After centrifugation at 500 × *g* for 3 min single cells were seeded into cell culture dishes. After 48 h of incubation the number of colonies and single cells were determined.

2.5. Ca^{2+} imaging and confocal laser scanning microscopy

$[Ca^{2+}]_i$ was monitored using the fluorescent dye fluo-3, AM. Multicellular spheroids were mounted to poly-L-lysine (Sigma, Deisenhofen, Germany) coated coverslips and were subsequently loaded for 60 min in F10 cell culture medium with 10 μM fluo-3, AM, dissolved in dimethyl sulfoxide (final concentration 0.1%) and pluronic F-127 which facilitates the solubilization of fluo-3, AM (final concentration < 0.025%). After loading, the spheroids were rinsed three times in E1 buffer containing (in mM) NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, HEPES 10 (pH 7.4 at 37°C). Superfusion was performed by gravity at a rate of 10 ml/min. A 90% volume exchange was achieved within 10 s. The experiments were performed at 37°C. Fluorescence data were recorded using an inverted confocal laser-scanning microscope (LSM 410; Zeiss, Jena, Germany) equipped with a 25× objective numerical aperture 0.85 (Neofluar, Zeiss). Fluorescence was excited by the 488 nm line of an argon-ion laser. Emission was recorded using a LP 515 nm filter set. Processing of images (512 × 512 pixels, 8 bit) was carried out by the Time-software facilities of the confocal setup. Full-frame images were acquired and stored automatically at 2 s intervals to a 16-megabyte video memory of the confocal setup. The minimum, maximum, mean, standard deviation, and integrated sum of the pixel values in a region of interest (selected using an overlay mask) were written to a data file and routinely exported for further analysis to the commercially available Sigma Plot (Jandel Scientific, Erkrath, Germany) graphic software. Because fluo-3 does not permit the use of ratio measurements, data are presented in arbitrary units as percentage of fluorescence variation (F/F_0) with respect to the resting level F_0.

2.6. Immunohistochemical techniques

The c-fos (AB-2) polyclonal antibody (5 μg/ml) was obtained from Calbiochem (Cambridge, USA). c-fos antibody staining was performed on whole mount MCS. The secondary antibody was a Cy3 labelled goat anti-rabbit IgG (H+L) antibody (concentration 1.2 mg/ml) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1/400. Excitation was performed using a 543 nm helium-neon laser of the confocal setup. Emission was recorded using a LP 570 nm filter set.

2.7. Statistical analysis

Data are given as mean values ± S.E., with *n* denoting the number of experiments. Student's *t*-test for unpaired data was applied as appropriate. A value of $P \leq 0.05$ was considered significant.

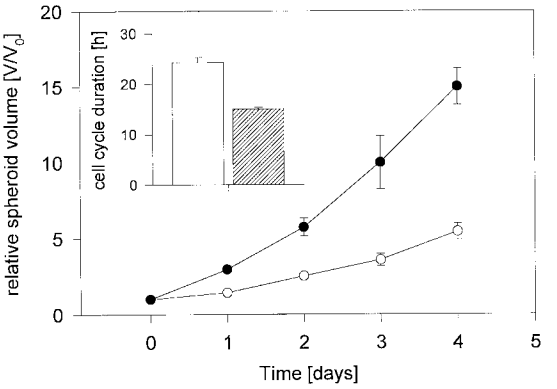

Fig. 1. Growth kinetics of multicellular tumor spheroids, 100 ± 30 μm in diameter which were incubated for 1 h with H_2O_2 (filled circles). Control, without treatment (open circles). The insert shows the cell cycle duration of control cells (open bar) and cells treated for 1 h with 20–100 nM H_2O_2 (hatched bar).

3. Results

3.1. Effect of exogenously added H_2O_2 on the growth and cell cycle kinetics of multicellular tumor spheroids

As shown in Fig. 1 nanomolar (20–100 nM) concentrations of H_2O_2 enhanced the growth kinetics of multicellular tumor spheroids of 100 ± 30 μm in diameter. Spheroid growth was inhibited when H_2O_2 was applied at concentrations exceeding 1 μM (data not shown). The volume doubling time was shortened from 1.3 ± 0.2 days in control spheroids to 0.76 ± 0.2 days in treated spheroids ($n = 4$). To exclude the possibility that H_2O_2 induced cell swelling, spheroid volumes were determined 1 h after treatment with H_2O_2 and were not significantly different from volumes of control spheroids. In additional experiments, spheroids were enzymatically dissociated and cell volumes of single cells were determined. The cell volume of single cells amounted to 16.7 ± 2 μm ($n = 100$ cells) and did not significantly change after addition of 20–100 nM H_2O_2 to the incubation medium (data not shown).

It is known that small multicellular prostate tumor spheroids (diameter 100 ± 30 μm) predominantly consist of proliferating, cell cycle active cells [20]. The enhancement of tumor growth should therefore be due to an acceleration of the cell cycle. To evaluate the duration of the cell cycle, control as well as H_2O_2-treated spheroids were enzymatically dissociated and colonies forming within 48 h from single cells were evaluated. The duration of the cell cycle was significantly ($P \leq 0.05$) shortened from 24.3 ± 1.7 h to 15 ± 0.6 h in control and treated spheroids, respectively ($n = 3$).

3.2. H_2O_2 increases c-fos expression in multicellular tumor spheroids

Any mitogenic stimulus should be correlated with the expression of growth-related genes of the early immediate response family. As shown in Fig. 2A, B incubation of multicellular tumor spheroids with 100 nM H_2O_2 resulted in a transient expression of c-fos as revealed by immunohistochemistry. c-fos expression was maximal 1 h after addition of H_2O_2. At the maximum the control level was increased by a factor of 3.3 ± 0.3 ($n = 4$). Within 4 h c-fos was downregulated towards the resting level.

3.3. H_2O_2 releases Ca^{2+} from intracellular stores

$[Ca^{2+}]_i$ has been implicated in the control of several decisive steps of the cell cycle [21]. To evaluate a possible role of $[Ca^{2+}]_i$ in the H_2O_2-induced growth stimulation of multicellular prostate tumor spheroids, cells were loaded with fluo-3, AM and $[Ca^{2+}]_i$ was recorded as fluo-3 fluorescence. Fig. 3A shows that upon superfusion of spheroids with 20–100 nM H_2O_2 a transient rise of $[Ca^{2+}]_i$ occurred. Its maximum corresponded to 103 ± 9.4% of the resting $[Ca^{2+}]_i$. The duration of the $[Ca^{2+}]_i$ transient was 69.3 ± 9.5 s ($n = 6$).

To address the source of the $[Ca^{2+}]_i$ transient spheroids were superfused with low (10 nM) $[Ca^{2+}]_o$ medium. Under these conditions, H_2O_2 induced a $[Ca^{2+}]_i$ transient which was not significantly different from control in amplitude and duration (maximum corresponding to 110 ± 19% of the resting $[Ca^{2+}]_i$, duration corresponding to 68.9 ± 4 s), indicating that the $[Ca^{2+}]_i$ transient was predominantly mediated by a Ca^{2+} release from intracellular stores ($n = 6$) (Fig. 3B). The difference in shape of the $[Ca^{2+}]_i$ transient was presumably due to an additional Ca^{2+} influx component under control conditions which was absent under low $[Ca^{2+}]_o$ conditions. The $[Ca^{2+}]_i$ transient was totally abolished when intracellular Ca^{2+} stores were depleted previous to the addition of H_2O_2. The depletion of stores was accomplished either by thapsigargin (Fig. 3C) ($n = 3$), which releases Ca^{2+} from intracellular stores and blocks the endoplasmic reticulum Ca^{2+}-ATPase, or by chelation of $[Ca^{2+}]_i$ by intracellular BAPTA ($n = 3$) (Fig. 3D).

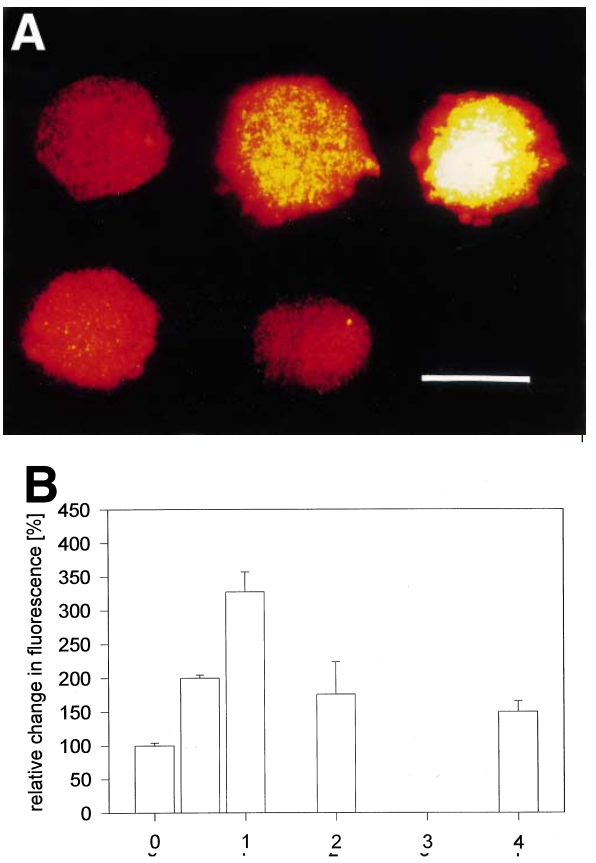

Fig. 2. Immunostaining of c-fos expression in multicellular tumor spheroids which were treated for 1 h with 20–100 nM H_2O_2. A: c-fos immunostaining of representative spheroids. From the upper left to the lower right: control, 0.5 h, 1 h, 2 h, 4 h after addition of H_2O_2 to the incubation medium. Focus is on the surface of spheroids (bar = 100 μm). B: Relative fluorescence increase (%) of c-fos cross-reactivity at different time points after exposure to H_2O_2. Control (1 and 2 antibody) was set to 100%.

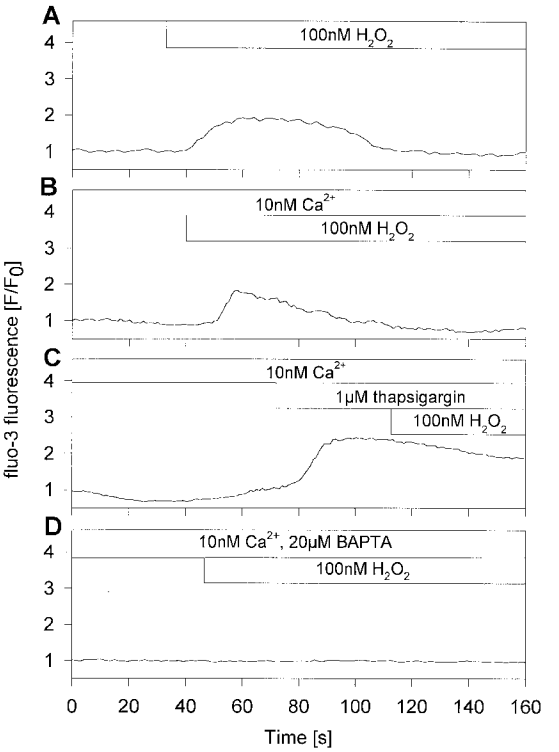

Fig. 3. Intracellular changes in $[Ca^{2+}]_i$ upon treatment of multicellular tumor spheroids with 20–100 nM H_2O_2. A: In the presence of 1.8 mM extracellular Ca^{2+}. B: Under low (10 nM) Ca^{2+} conditions. C: In the presence of 1 μM thapsigargin. D: Under low (10 nM) Ca^{2+} conditions after preincubation for 30 min with 10 μM BAPTA, AM. Representative tracings.

3.4. Possible involvement of growth factor receptors in the H_2O_2-induced $[Ca^{2+}]_i$ transient

Mitogen-stimulated signal transduction pathways are initiated through the interaction of cytokines with the respective receptors. Many of these cytokine receptors have been demonstrated to be additionally activated by sulfhydryl oxidation [22]. To assess a possible activation of growth factor receptors by H_2O_2, tumor spheroids were preincubated with either 0.1 mM suramin or 0.1 mM basilen blue which have previously been demonstrated to inhibit ATP-induced Ca^{2+} mobilization via the P_2-purinoreceptor and to inhibit ERK2 activation by H_2O_2 [13]. Fig. 4A shows that suramin pretreatment inhibited the $[Ca^{2+}]_i$ transient observed after addition of H_2O_2 to the incubation medium. After washout of suramin, the H_2O_2-induced $[Ca^{2+}]_i$ transient fully recovered with a maximum corresponding to 112% ± 8 of the resting $[Ca^{2+}]_i$ ($n = 4$). After preincubation of spheroids with basilen blue the H_2O_2-induced $[Ca^{2+}]_i$ transient was irreversibly blocked ($n = 8$) (Fig. 4B). These data indicate that H_2O_2 may mimic the action of agonist binding to growth factor receptors, presumedly ATP binding to the P_2-purinoreceptor in the plasma membrane.

3.5. Effects of receptor inactivation and $[Ca^{2+}]_i$ chelation on growth kinetics of multicellular tumor spheroids and c-fos expression

If the $[Ca^{2+}]_i$ transient observed after addition of H_2O_2 to the incubation medium is a determinant of the observed

growth stimulation of multicellular tumor spheroids, inhibition of the $[Ca^{2+}]_i$ signal should likewise inhibit the H_2O_2-induced growth stimulation and c-fos expression. Fig. 5A shows that after either suramin (0.1 mM) and basilen blue (0.1 mM) treatment or chelation of $[Ca^{2+}]_i$ during the incubation period of multicellular tumor spheroids with H_2O_2, the observed growth kinetics were not significantly different from control. Suramin and basilen blue treatment or chelation of $[Ca^{2+}]_i$ for 1 h in the absence of H_2O_2 was without any effect on spheroid growth (data not shown). Fig. 5B shows that after either suramin or basilen blue treatment or chelation of $[Ca^{2+}]_i$ c-fos expression after H_2O_2 exposure was not significantly different from control, which altogether indicates that H_2O_2 exerts its cell cycle-stimulatory effect via a Ca^{2+}-signaling pathway.

4. Discussion

The present study was undertaken to evaluate the signal transduction underlying the growth stimulation of multicellular prostate tumor spheroids by H_2O_2. The elucidation of the mechanisms by which a prooxidant state modulates gene expression is a major goal in cancer research. There is now significant evidence for a role of ROS as signaling substances which may utilize the same signal transduction pathway/s as the cytokines and mitogens [3]. Many cell types including cancer cells are provided with mechanisms to endogenously produce ROS which may be involved in triggering the cell cycle. On the other hand, many antioxidants have been shown to be anticarcinogenic and inhibit cell proliferation [23].

In the present study we used multicellular tumor spheroids in vitro which mimic the in vivo situation of micrometastases and avascular regions of solid tumors. With increasing growth, these spheroids develop extended areas of quiescent cells arrested in the G_0/G_1 phase of the cell cycle and a central necrosis. For our experiments small tumor spheroids were selected which predominantly consist of proliferating cells [20]. Our data demonstrate that nanomolar concentrations of H_2O_2 enhance tumor growth due to an acceleration of the cell cycle. H_2O_2 elicited a transient rise in $[Ca^{2+}]_i$ which was predominantly due to Ca^{2+} release from intracellular

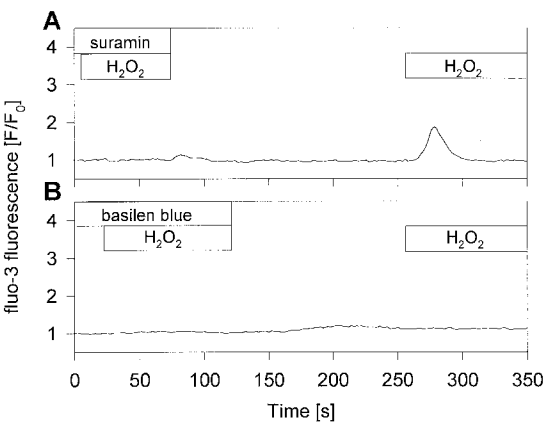

Fig. 4. Effect of growth factor receptor inactivation by either suramin (A) or basilen blue (B) on intracellular $[Ca^{2+}]_i$ changes upon H_2O_2 (100 nM) treatment. Spheroids were preincubated with the agents for 30 min. Note that H_2O_2 elicited a $[Ca^{2+}]_i$ transient not significantly different from control after washout of suramin. Representative tracings.

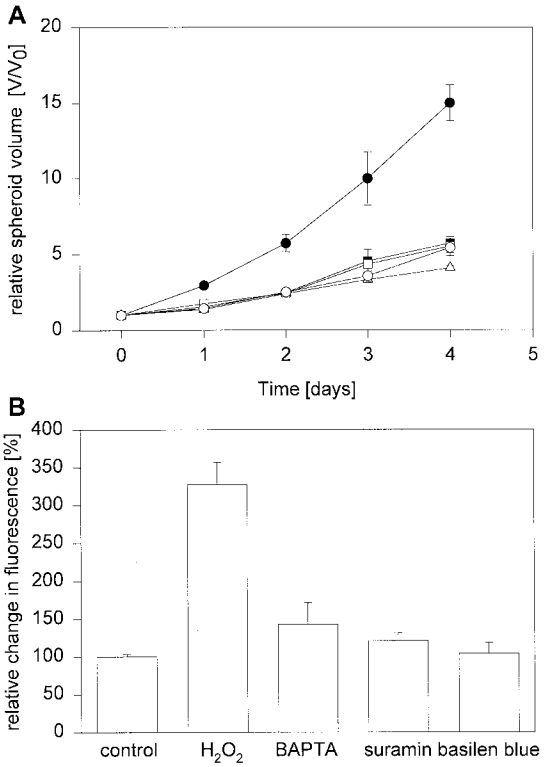

Fig. 5. A: Effect of $[Ca^{2+}]_i$ chelation (filled squares), suramin (open squares) and basilen blue (open triangles) treatment on the H_2O_2-induced growth stimulation of multicellular tumor spheroids. The agents were applied during the 1 h incubation of spheroids with 20–100 nM H_2O_2. Control, H_2O_2-treated (filled circles) and untreated (open circles) spheroids. B: Effect of $[Ca^{2+}]_i$ chelation, suramin and basilen blue treatment on H_2O_2-induced c-fos immunoreactivity. The data show the relative change in fluorescence (%) as compared to control (1 and 2 antibody) which was set to 100%. The data represent c-fos immunoreactivity 1 h after addition of H_2O_2 to the incubation medium.

stores as it was still present in low $[Ca^{2+}]_o$ solution but was abolished when either $[Ca^{2+}]_i$ was chelated by intracellular BAPTA or intracellular Ca^{2+} stores were depleted by the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin. Similar $[Ca^{2+}]_i$ transients have been observed upon receptor binding of mitogens with subsequent activation of mitogen-activated protein kinase pathways and early immediate response genes such as c-fos, c-jun, c-myc, and egr-1 [24–26]. Our experiments demonstrate that the P_2-purinoreceptor blockers suramin and basilen blue [27,28] abolished the H_2O_2-induced $[Ca^{2+}]_i$ transient. The presence of purinergic receptors has been recently demonstrated in the Du-145 prostate cancer cell line used in the present study [29] and a mitogenic stimulation of cells by ATP has been shown for several preparations [30–32]. In thyroid FRTL-5 cells and vascular smooth muscle cells this mitogenic stimulation was accompanied by MAPK and c-fos activation [25,33]. Our data exclude an action of H_2O_2 via receptor tyrosine kinase activation which has been demonstrated for Jurkat cells [17] as genistein, a potent tyrosine kinase antagonist at a concentration of 10-100 μM failed to inhibit the observed $[Ca^{2+}]_i$ transient, c-fos activation and growth stimulation of multicellular spheroids (data not shown). Hence the data of the present study suggest that the effect of H_2O_2 on cell cycle stimulation of cells within multicellular spheroids is presumedly propagated via activa-

tion of the P_2-purinoreceptors and subsequent $[Ca^{2+}]_i$ mobilization but not via direct interaction with intracellular Ca^{2+} stores. A utilization of $[Ca^{2+}]_i$ from ATP-sensitive stores by H_2O_2 in an endothelial cell preparation has been recently demonstrated [34]. H_2O_2 thus mimics the action of growth factors as has been suggested previously [13].

Our data further show that H_2O_2 transiently induced c-fos expression in multicellular tumor spheroids. Expression of the protooncogene c-fos has previously been reported to be crucial for cell cycle activation and cell proliferation [35–37]. To test whether the activation of c-fos observed in the present study was due to a direct redox activation of gene transcription rather than a $[Ca^{2+}]_i$-related phenomenon, the $[Ca^{2+}]_i$ transient elicited by H_2O_2 was inhibited by either suramin and basilen blue treatment or $[Ca^{2+}]_i$ chelation by intracellular BAPTA. We were able to demonstrate that any inhibition of the $[Ca^{2+}]_i$ transient upon H_2O_2 treatment blunted the observed increase in c-fos expression. Preincubation of tumor spheroids with suramin, basilen blue and BAPTA prior and during exposure to H_2O_2 likewise inhibited the observed growth effect, which indicates that the H_2O_2-induced growth stimulation of multicellular spheroids is closely linked to the $[Ca^{2+}]_i$ transient upon H_2O_2 treatment.

H_2O_2 readily permeates cell membranes. Intracellularly generated H_2O_2 may thus penetrate the plasma membrane and from outside activate receptors inducing signal transduction pathways that are normally used by growth factors and cytokines. The anticarcinogenic and antiproliferative action of several antioxidants may be due to an extracellular scavenging of ROS before they are able to promote tumor growth by stimulation of mitogen-activated signal transduction pathways via growth factor receptor activation.

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