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Cyclosporin A generates superoxide in smooth muscle cells

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Abstract
Cyclosporin A (CsA) generates superoxide in smooth muscle cells. Our earlier studies have demonstrated that the increase in the vasopressin type 1 receptor induced in vascular smooth muscle cells in the presence of CsA is probably due to superoxide (Krauskopf et al., J Biol Chem 278, 41685–41690, 2003). This increase in vasopressin receptor is likely at the base of increased vascular responsiveness to vasoconstrictor hormones and hypertension induced by CsA. Here, we demonstrate that CsA produces superoxide. In addition, our data show that superoxide generation does not originate from the major cellular superoxide generating systems NAD(P)H oxidase or xanthine oxidase. Our results suggest that the side effects of CsA could be diminished with the help of SOD mimetic drugs.

Keywords: *Cyclosporin A, superoxide, superoxide dismutase, NAD(P)H oxidase*

Introduction

A large number of transplant patients are treated with the immunosuppressive drug cyclosporin A (CsA) to prevent graft rejection [1]. In addition, CsA is also used to treat various autoimmune diseases [1]. Unfortunately, the use of this drug is limited because of the development of considerable side effects in most patients of which hypertension and nephrotoxicity are most relevant [2,3]. Drug-induced vasoconstriction is likely to be at the origin of both the hypertensive and nephrotoxic side effects of CsA [4,5]. Evidence points to a CsA-induced increase in contraction of vascular and renal smooth muscle/mesangial cells [4–10].

We and others have shown previously that CsA caused an elevation in cytosolic calcium concentrations and an exaggerated contractile response in vascular smooth muscle and mesangial cells [7,11,12] and in vessels treated *in vitro* [13] when these were stimulated with vasoconstrictor hormones, such as noradrenalin, angiotensin II, endothelin-1, serotonin or [Arg^8]-vasopressin (AVP). In support of a role for cytosolic calcium, it has been demonstrated that the administration of calcium channel blockers attenuated both renal injury and hypertension induced by CsA [14]. Further, we and others have shown that CsA increased the response to vasoconstrictor hormones by augmenting the membrane expression of several vasoconstrictor hormone receptors (Ang II, AVP, ET-1) both in animals [15,16] and in cellular models [17–19]. Later, we have shown that the upregulation of the vasopressin receptor was due to stabilization of the corresponding mRNA in rat aortic smooth muscle cells (RASMC) [20]. And recently, we have demonstrated that reactive oxygen species (ROS) generated in the presence of CsA were responsible for the increase in vasopressin receptor expression in RASMC [21].

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Several research groups including ours have also shown that CsA is able to generate ROS, most likely superoxide [22–25]. In this work, we present a more detailed analysis of CsA-induced superoxide generation in RASMC. Further, we show that CsA-induced superoxide generation occurred intracellularly and does not originate from the major cellular superoxide generating systems NAD(P)H oxidase or xanthine oxidase. In an earlier report, we have already shown that CsA does not generate ROS by the cytochrome P450 system often proposed as a source of CsA-induced ROS formation [25]. Our findings that the side effects of CsA seem to be mediated by superoxide open the possibility for co-treatment of CsA with low-molecular weight SOD mimetic drugs that are under development to limit these side effects.

Materials and methods

Chemicals and buffers

CsA was a gift from Novartis Pharma (Basel, Switzerland). Fetal calf serum (FCS), was from Invitrogen (Basel, Switzerland). Ciproxin was purchased from Bayer Pharma AG (Zürich, Switzerland), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and dihydroethidium (DHE) from Molecular Probes (Juro Supply AG, Lucerne, Switzerland). Dulbecco's modified Eagle medium (DMEM), superoxide dismutase, hypoxanthine and xanthine oxidase were from Sigma (Buchs, Switzerland). Stock solutions of chemicals were prepared in ethanol or buffer. The CsA stock solution was prepared in ethanol at a concentration of 1 or 10 mM and diluted to either 1 or 10 μM for experiments, respectively, thus resulting in maximal final ethanol concentration of 0.1%. Fresh CsA stock solutions were prepared every week. DCFH and DHE stock solutions were prepared in ethanol. In all experiments, 0.1% ethanol served as control and was set at 100%. The physiological salt solution (PSS) contained 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5 mM Hepes and 10 mM glucose, adjusted to pH 7.6.

Cell cultures

Rat aortic smooth muscle cells (RASMC) were prepared from aortae of male Wistar Kyoto rats (200–300 g) as described [13]. RASMC were seeded at a density of 3000 cells/well into 96-well plates in Dulbecco's modified Eagle medium (DMEM) supplemented with essential amino acids, vitamins, 0.001% ciproxin and 10% FCS. Cells were kept at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were used at confluence (after 7–9 days in culture) between passages 6 and 11. Twenty-four hours before experiments, culture media were replaced with fresh DMEM without FCS. Treatment of RASMC with CsA and other agents was always performed in serum-free DMEM.

Determination of ROS generation

The fluorescent probes 2′,7′-dichlorofluorescin diacetate (DCFH-DA) or DHE were used to determine intracellular ROS and $O_2^{-\cdot}$ formation, respectively, as described [25]. Briefly, DCFH-DA is deacetylated by cellular esterases to yield cell-entrapped DCFH competent to react with ROS. Reaction of DCFH with ROS will oxidize DCFH to the fluorescent 2′,7′-dichlorofluorescein (DCF). In a biological milieu where peroxidases and metals are present, DCFH can be used to detect hydrogen peroxide, hydroxyl radicals or peroxynitrite, but DCFH is not able to detect superoxide [26,27]. Cytosolic DHE exhibits blue fluorescence; however, once this probe is oxidized to ethidium, it intercalates with cellular DNA resulting in bright red fluorescence. Confluent RASMC cultures were incubated in DMEM without FCS in the presence of 10 μM DCFH-DA or 10 μM DHE and CsA or the compounds to be tested for 1 h at 37°C. After incubation, the medium was replaced with PSS and cellular fluorescence (DCFH: $\lambda_{ex} = 485$ nm and $\lambda_{em} = 520$ nm, DHE: $\lambda_{ex} = 544$ nm and $\lambda_{em} = 612$ nm) was determined at 37°C in a microtiter plate reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany). Data are expressed as percent of control values because of inter-experimental variations. Due to the variability between experiments, the gain applied to the photomultiplier tube was adjusted before each experiment.

Data analysis

Results are presented as the means of at least three independent experiments with bars indicating standard errors of the mean (SEM). Statistical evaluation was performed by one-way ANOVA followed by Newman Keuls or Dunnett post-tests using the software lnPlotPrism (GraphPad Software, San Diego, USA). Differences with a p value < 0.05 were considered significant.

Results and discussion

Our earlier data were generated using the indirect ROS detector DCFH in combination with selective radical scavengers to specifically detect superoxide in RASMC. Although many studies have used lucigenin-enhanced luminescence as specific detector of superoxide, there is a large body of literature demonstrating that due to its redox-cycling potential in biological samples linked to superoxide generation this probe is not adequate for the detection of

superoxide [28–32]. We have also tried other probes such as 2-methyl-6-(4-methoxyphenyl)-3,7-dihydro-imidazo(1,2-α)pyrazine-3-one (MCLA) and coelenterazine. However, we found that MCLA did not readily cross the plasma membrane and, for an unknown reason, coelenterazine did not result in an increased luminescent signal with a control system of hypoxanthine and xanthine oxidase alone. Therefore, we tried the recently described selective $O_2^{-\cdot}$ detector dihydroethidium [33–35]. Figure 1 shows that CsA significantly increases ROS and $O_2^{-\cdot}$ generation in a concentration-dependent manner in RASMC. For further experiments, DCFH was used as it produced a more robust fluorescent signal.

Figure 2 shows that CsA is able to generate $O_2^{-\cdot}$ intracellularly as extracellularly added SOD or catalase (CAT) had no effect on the DCF fluorescent signal induced by CsA. Given that extracellularly added enzymes cannot penetrate into cells it was expected that CAT would have no effect. Even when extracellularly added CAT was increased to 1000 U/ml

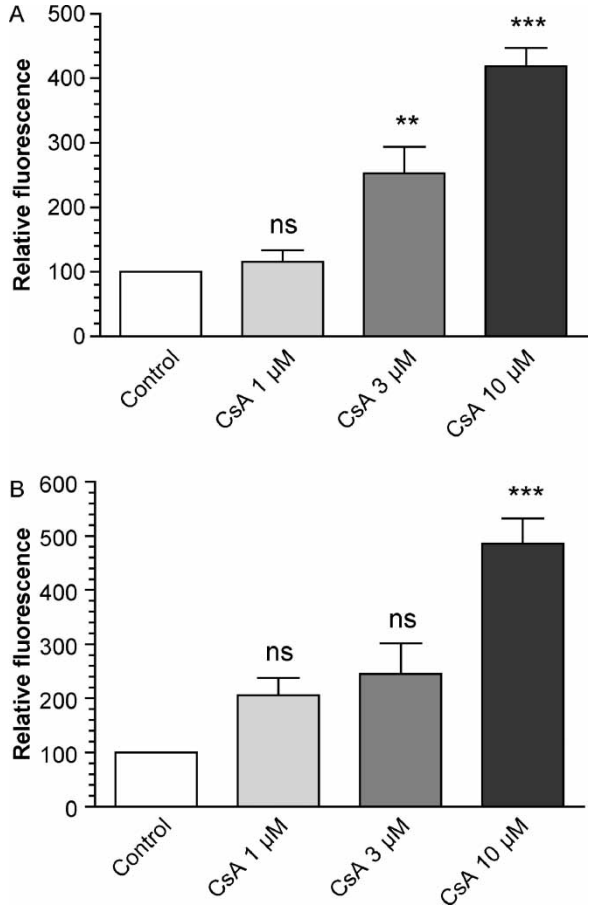

Figure 1. ROS formation by CsA. RASMC were treated at 37°C with CsA at the indicated concentration for 1 h in the presence of either 10 μM DCFH (A) or 10 μM DHE (B). Data are means ± SEM of at least three independent experiments performed in triplicate. Asterisks indicate values significantly different from CsA at $p < 0.01$ (**) or at $p < 0.001$ (***), ns, statistically not significant.

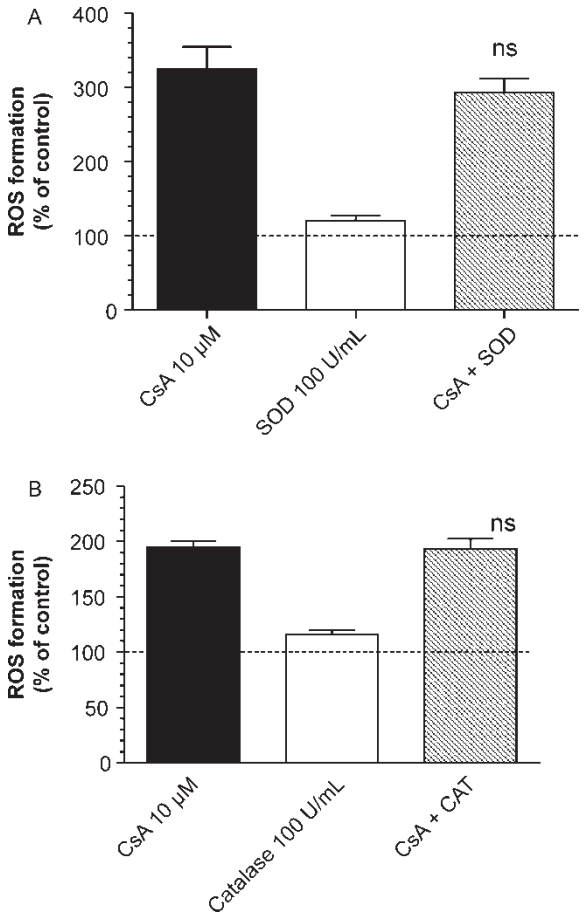

Figure 2. Effect of SOD and CAT on CsA-mediated increase in ROS generation. RASMC were treated at 37 °C with SOD (100 U/ml) or CAT (100 U/ml) in culture medium without serum in the presence or absence of CsA (10 μM) and DCF-mediated fluorescence was determined 1 h later. Data are mean ± SEM of at least three independent experiments performed in triplicate, ns, statistically not significant.

we were unable to observe any interference with CsA-mediated DCF formation (not shown). This is in contrast to published data where extracellularly added CAT was able to influence intracellular signaling [36]. Because catalase is not able to penetrate into cells the inhibitory effect of extracellularly added CAT in these experiments can only be explained by an obligatory extracellular presence of ROS in the signal transduction pathway described in the paper by Sundaresan et al. [36] which was not the case in our study. It should be noted that Wolf et al. have observed that contraction of glomerular rings was inhibited by SOD but also by CAT [37] however, the same reservations apply as these enzymes are unable to penetrate into cells under normal conditions.

We have shown earlier [21] that if cells were pre-treated with 100 U polyethylene glycol coupled SOD (PEG-SOD) for 6 h followed by washing and removal of extracellular PEG-SOD, the CsA-induced DCF signal was increased by approximately 30%. On the other hand, when cells were pre-treated with 300 U

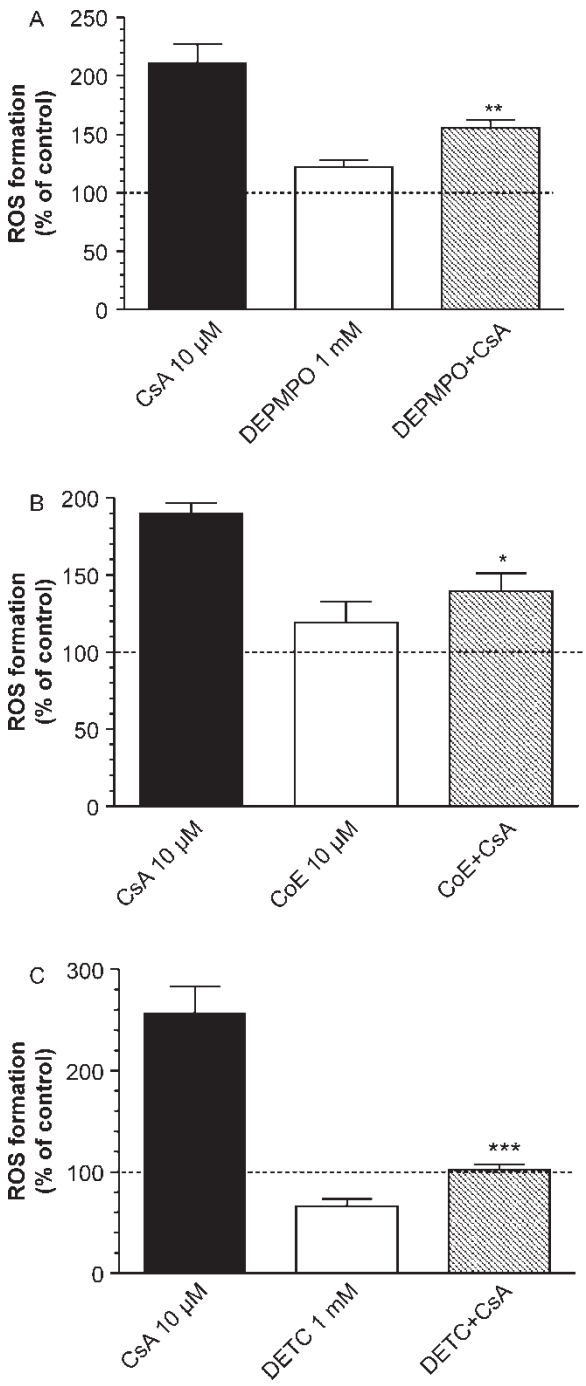

Figure 3. Effect of superoxide scavengers on CsA-mediated increase in ROS generation. RASMC were treated at 37°C with DEPMPO (1 mM, A), coelenterazine (CoE, 10 µM, B) or DETC (1 mM, C) in culture medium without serum in the presence or absence of 10 µM CsA and DCF-mediated fluorescence was determined 1 h later. Data are mean ± SEM of at least three independent experiments performed in triplicate. Asterisks indicate values significantly different from CsA at $p < 0.05$ (*), $p < 0.01$ (**) or at $p < 0.001$ (***).

polyethylene glycol coupled catalase (PEG-CAT) for 6 h the CsA-induced DCF signal was decreased by approximately 30%. It should be noted that neither PEG-SOD nor PEG-Cat alone had any effect on basal DCF fluorescence (not shown). Since DCFH only detects intracellular ROS (mostly H_2O_2) because it is trapped within the cells, this confirms the $O_2^{-\cdot}$ production induced by CsA yielding higher H_2O_2 levels in the presence of higher SOD levels. On the other hand, increased cellular levels of CAT decreased the DCF signal due to increased reduction of H_2O_2 to H_2O.

To further investigate whether CsA was able to generate $O_2^{-\cdot}$ intracellularly, we used selective superoxide scavengers. These included the $O_2^{-\cdot}$-selective spin trapping agent 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO). This probe has been shown to react with $O_2^{-\cdot}$, but also with alkyl and alkoxyl radicals [29,38,39]. However, it was not shown to react with H_2O_2, hydroxyl radicals or peroxynitrite. The other scavenger used was coelenterazine [40]. Because it has been described to react with $O_2^{-\cdot}$ which would suggest that it could also scavenge $O_2^{-\cdot}$. As shown in Figure 3(A,B), both of these agents were able to attenuate the CsA-induced increase in DCF fluorescence. As these two agents are in competition for the dismutation reaction (spontaneous or SOD-catalyzed), it was expected that the signal could not be completely blocked. Because both compounds are relatively selective for superoxide, these results further support the finding that CsA was able to generate superoxide in the intracellular space.

We then tested whether inhibition of Cu/Zn-SOD with diethyldithiocarbamate (DETC) would be able to block the CsA-induced increase in DCF fluorescence. DETC is a Cu^+ chelator that is able to extract Cu^+ from the active site of Cu/Zn-SOD, thereby inactivating it. Figure 3C shows that indeed DETC was able to completely block the DCF signal induced by CsA. Moreover, it also decreased the basal level of H_2O_2 detected with DCFH indicating that DETC was able to decrease H_2O_2 levels resulting from $O_2^{-\cdot}$ dismutation from endogenous sources. The residual H_2O_2 detected by DCFH may be the result of spontaneous dismutation.

We have shown previously [21] that $O_2^{-\cdot}$ was able to increase the expression of the type 1A vasopressin receptor. Under conditions where Cu/Zn-SOD was inhibited with DETC one would expect that $O_2^{-\cdot}$ would accumulate. Indeed, treatment of RASMC with DETC resulted in a stimulation of the V_{1A}-receptor expression under both basal and CsA-stimulated conditions [21]. However, since DETC is a copper–zinc chelator, it can also react with other metalloenzymes that are often involved in redox reactions. It is interesting to note that graded inhibition of Cu/Zn-SOD with DETC resulted in a graded increase in $O_2^{-\cdot}$ levels that either caused growth stimulation or induced apoptosis at low or high $O_2^{-\cdot}$ levels, respectively [41].

Next, the question of the origin of the CsA-induced $O_2^{-\cdot}$ generation was addressed. We have already shown that a possible source that has frequently been

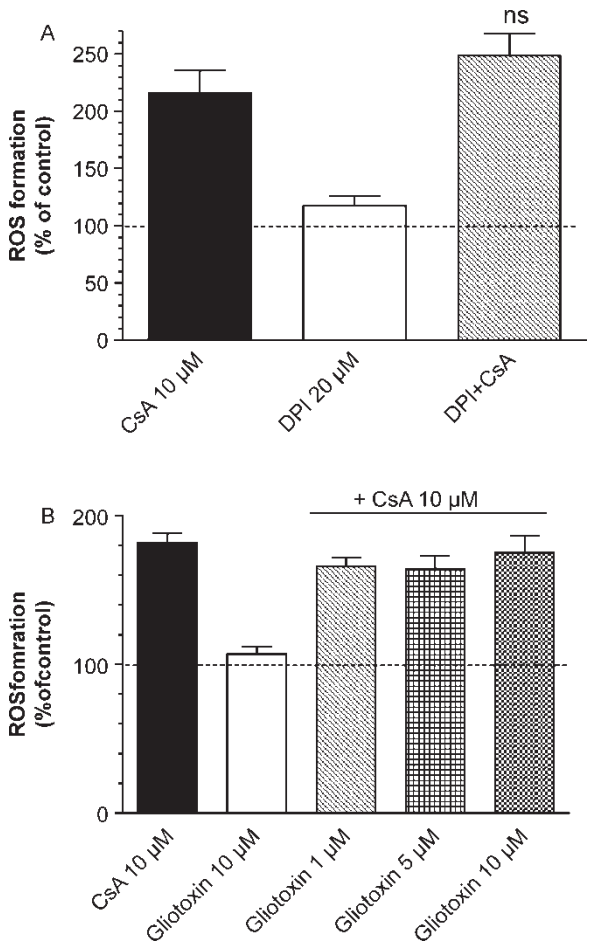

Figure 4. Effect of NAD(P)H oxidase inhibitors on CsA-mediated increase in ROS generation. RASMC were treated at 37°C with DPI (20 μM, A) or increasing concentrations of gliotoxin (B) in culture medium without serum in the presence or absence of CsA (10 μM) and DCF-mediated fluorescence was determined 1 h later. Data are mean ± SEM of at least three independent experiments performed in triplicate, ns, statistically not significant.

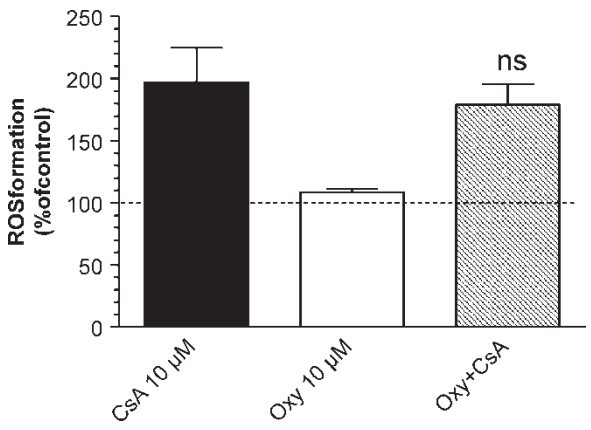

Figure 5. Effect of oxypurinol on CsA-mediated increase in ROS generation. RASMC were treated at 37°C with oxypurinol (10 μM) in culture medium without serum in the presence or absence of CsA (10 μM) and DCF-mediated fluorescence was determined 1 h later. Data are mean ± SEM of at least three independent experiments performed in triplicate, ns, statistically not significant.

suggested as the source of CsA-mediated ROS formation, the cytochrome P450 system, could be excluded as the source of ROS formation [25]. The most likely cellular source for ROS formation in non-phagocytic cells is the non-neutrophil NAD(P)H oxidase (NOX) system that is present in most, if not all, cells [42]. This system has been implicated in cellular $O_2^{-\cdot}$ generation under many pathological conditions especially of the cardiovascular system [43]. The major NOX component in non-phagocytic cells is a 65 kDa flavin-containing protein called NOX1 (or MOX1) that shuttles electrons from NAD(P)H to oxygen and is inhibited by the general FAD binding agent diphenylene iodonium (DPI) [42]. However, Figure 4 shows, that the CsA-generated $O_2^{-\cdot}$ detected as DCF fluorescence is not affected by DPI. To confirm this observation, we also used the selective NOX inhibitor gliotoxin [44]. In addition to NOX inhibition, other activities ascribed to gliotoxin include the inhibition of protein farnesyltransferase activity [45], non-competitive inhibition of Ras [46] or activation of the ryanodine receptor [47]. To activate the ryanodine receptor via thiol oxidation relatively high gliotoxin concentrations were needed (>200 μM) [47]. The other activities of gliotoxin could converge onto its inhibition of protein farnesyltransferase activity. Ras farnesylation serves to anchor this protein in the plasma membrane and inhibition of its farnesylation will probably lead to its mis-localization and/or degradation. Further, it seems that Ras is essential for NOX activation. In fact, it is likely that the growth promoting activity of mutated Ras may be mediated by increased $O_2^{-\cdot}$ generation [48]. Therefore, inhibition of Ras farnesylation will probably lead to inhibition of NOX activity and consequently to inhibition of NOX-dependent $O_2^{-\cdot}$ generation. However, because of its activity on the inhibition of protein farnesylation and as thiol oxidizing agent, gliotoxin may have other cellular activities. Nevertheless, we used it as a selective inhibitor of NOX activity at low concentrations [44]. As shown in Figure 4B, gliotoxin at 1, 5, 7 or 10 μM had no effect on CsA-induced radical formation, in further support that NOX is not implicated in CsA-induced $O_2^{-\cdot}$ generation.

Another important ROS generating system is xanthine oxidase (XO) [49], a system that is activated under hypoxic conditions. Under normal conditions this enzyme of purine degradation is present as xanthine dehydrogenase that transforms purines to xanthine, then to hypoxanthine and finally to uric acid. In this reaction, electrons are transferred to NAD^+. However, under hypoxic or oxidative stress conditions xanthine dehydrogenase is converted into XO by thiol oxidation and/or proteolytic cleavage. Now XO transfers electrons to O_2 instead of NAD^+ to generate superoxide. The xanthine analogue

allopurinol and its degradation product oxypurinol serve as specific inhibitors of XO. Figure 5 shows that treatment of RASMC with oxypurinol had no effect on the CsA-mediated $O_2^{-\cdot}$ generation, thus excluding a contribution of XO in CsA-mediated $O_2^{-\cdot}$ formation.

After the three major cellular ROS generating systems could be excluded as sources for CsA-mediated $O_2^{-\cdot}$ formation, further trials to identify the source of CsA-mediated $O_2^{-\cdot}$ formation were also unsuccessful. Neither nitric oxide synthase nor cyclo-oxygenase nor lipoxygenases appeared to mediate CsA-dependent ROS formation (not shown). The tests using different mitochondrial inhibitors (i.e. respiratory chain, adenosine nucleotide translocase, ATPase) were inconclusive. Thus, we were unable to identify the source of CsA-mediated $O_2^{-\cdot}$ generation. In the discussion of a recent publication, Navarro-Antolin et al. suggested that CsA might inhibit mitochondrial Mn-SOD [23]. If CsA also inhibited cytosolic Cu/Zn-SOD one would expect $O_2^{-\cdot}$ levels to rise. However, we detected an increase in $O_2^{-\cdot}$ levels in the presence of CsA also by the means of the fluorescent probe DCFH that detects its dismutation product H_2O_2. Thus, it appears very likely that CsA is not able to increase $O_2^{-\cdot}$ levels by inhibiting Cu/Zn-SOD.

In summary, our results demonstrate that CsA is able to generate $O_2^{-\cdot}$ in vascular smooth muscle cells. Even though we have not been able to identify by what mechanism CsA is able to increase $O_2^{-\cdot}$ levels, the fact that CsA-mediated $O_2^{-\cdot}$ production could be linked to its side effects [21] suggests that specific intervention strategies could be employed to limit these side effects. As our results have demonstrated that increasing intracellular SOD activity with PEG-SOD was able to decrease CsA-induced $O_2^{-\cdot}$ levels and decrease V_{1A}-receptor expression levels [21], we propose that the CsA-induced side effects could be limited with the use of SOD mimetic drugs. In fact, the group of Thurman has shown that viral delivery of SOD was able to reduce CsA-induced nephrotoxicity [50] which shows that SOD treatment may also work *in vivo*. Although SOD mimetics seem to be useful for the treatment of various diseases [51], none of these low molecular weight SOD mimetics are yet available as approved drugs. But in view of their medical potential they will probably soon reach the market. It is likely that such drugs may also be useful to limit the CsA-induced side effects and enhance the usefulness of this important immunosuppressive drug.

Acknowledgements

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