Mycophenolate Acid Inhibits Endothelial NAD(P)H Oxidase Activity and Superoxide Formation by a Rac1-Dependent Mechanism

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Abstract—Endothelial dysfunction precedes hypertension and atherosclerosis and predicts cardiac allograft vasculopathy and death in heart transplant recipients. Endothelial overproduction of reactive oxygen species, such as superoxide anions produced by NAD(P)H oxidase, induces endothelial dysfunction. Because immunosuppressive drugs have been associated with increased reactive oxygen species production and endothelial dysfunction, we sought to elucidate the underlying mechanisms. Reactive oxygen species, release of superoxide anions, and NAD(P)H oxidase activity were studied in human umbilical vein endothelial cells and in polymorphonuclear neutrophils. Gp91ds-tat was used to specifically block NAD(P)H oxidase. Transcriptional activation of different subunits of NAD(P)H oxidase was assessed by real-time RT-PCR. Rac1 subunit translocation and activation were studied by membrane fractionation and pull-down assays. Calcineurin inhibitors significantly increased endothelial superoxide anions production because of NAD(P)H oxidase, whereas mycophenolate acid (MPA) blocked it. MPA also attenuated the respiratory burst induced by neutrophil NAD(P)H oxidase. Because transcriptional activation of NAD(P)H oxidase was not affected, but addition of guanosine restored endothelial superoxide anions formation after MPA treatment, we speculate that the inhibitory effect of MPA was mediated by depletion of cellular guanosine triphosphate content. This prevented activation of Rac1 and, thus, of endothelial NAD(P)H oxidase. Because all heart transplant recipients are at risk for cardiac allograft vasculopathy development, these differential effects of immunosuppressants on endothelial oxidative stress should be considered in the choice of immunosuppressive drugs. (Hypertension. 2007;49:201-208.)

Key Words: endothelial cells • endothelial dysfunction • reactive oxygen species • immunosuppression • signal transduction

Endothelial dysfunction is an important risk factor for cardiovascular adverse events. Patients with autoimmune diseases, such as systemic lupus erythematosus, and organ transplant recipients bear an increased risk of coronary vascular dysfunction. Especially after heart transplantation, the development of cardiac allograft vasculopathy (CAV), which affects large epicardial arteries, as well as the microcirculation, remains a major drawback of long-term prognosis. On a molecular basis, the development of CAV includes an immune-mediated inflammatory response, including mononuclear cell infiltration, into allograft vessel walls by the recipient and vascular cytokine release leading to alteration of endothelial vasomotor function. In addition, classic risk factors of cardiovascular disease also foster CAV. Endothelial dysfunction occurs early after heart transplantation and might reflect CAV of the endothelium contributing to the development of CAV. Thus, the occurrence of coronary endothelial dysfunction in patients after heart transplantation has repeatedly been observed to predict the appearance and progression of CAV and of cardiac death. There are limited data regarding whether different immunosuppressive regimens after heart transplantation may have a significant impact on progression of CAV. It has been shown that the calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506), which cannot prevent CAV, can induce endothelial dysfunction and hypertension, which has been explained by an increase in vascular reactive oxygen species (ROS) formation. In contrast to this, the inhibitor of inosine monophosphate dehydrogenase (IMPDH), mycophenolate mofetil, or its active metabolite, mycophenolate acid (MPA), have been associated with positive effects on atherosclerosis or hypertension in animal models.
Considering the development and progression of CAV, observations of increased ROS formation caused by immunosuppressive drugs may be of high importance, because the endothelial function can be (at least in nontransplanted patients) profoundly altered by an increased cellular oxidative stress.\(^{19}\) This is likely to also be the case in transplant recipients, because a recent study shows that inhibition of the NO pathway worsens coronary endothelial dysfunction and intimal hyperplasia in graft coronary vasculopathy,\(^{20}\) suggesting that further decreasing vascular NO availability exerts detrimental effects in this disease setting. Superoxide anions (\(O_2^-\)) derived from NAD(P)H oxidase in endothelial cells rapidly inactivate NO within the endothelium, resulting in an impairment of NO-dependent vasoprotective effects.\(^ {19}\) It is now well understood that \(O_2^-\) production by this enzyme has a key role in the development of hypertension and pathogenesis of arteriosclerosis.\(^ {21,22}\) In addition, some drugs, which maintain endothelial function and prevent cardiovascular disease progression, partly exert their beneficial effects by inactivating endothelial NAD(P)H oxidase.\(^{23,24}\) However, it is not known whether an increased superoxide production by the endothelial NAD(P)H oxidase accounts for the endothelial dysfunction in CAV and how this is influenced by immunosuppressive drugs used to prevent graft rejection.

Therefore, we investigated the effects of various immunosuppressive compounds (CsA, FK506, and, particularly, MPA) on \(O_2^-\) formation and NAD(P)H oxidase in cultured human endothelial cells. In addition, we studied the mechanisms of altered NAD(P)H oxidase–dependent \(O_2^-\) production caused by these drugs.

**Methods**

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**Endothelial Cell Culture**

Primary human umbilical vein endothelial cells (HUVECs) were used as described.\(^{25}\)

**Drugs**

Clinically relevant drug doses were tested, which were 100 nmol/L (120 ng/mL) and 1 \(\mu\)mol/L (1202 ng/mL) for CsA; 100 nmol/L (82 ng/mL) and 1 \(\mu\)mol/L (822 ng/mL) for FK506; 10 nmol/L (9.6 ng/mL), 100 nmol/L (95.8 ng/mL), and 1 \(\mu\)mol/L (958 ng/mL) for rapamycin; and 1 \(\mu\)mol/L (433 ng/mL) and 10 \(\mu\)mol/L (4335 ng/mL) for MPA.

**Isolation of Human Polymorphonuclear Neutrophils**

Polymorphonuclear neutrophils (PMNs) were isolated from venous blood from healthy volunteers as described elsewhere.\(^ {26}\)

**Measurement of Superoxide Radicals and NAD(P)H Oxidase Activity**

For \(O_2^-\) measurements and assessment of reduced nicotinamide-adenine dinucleotide–dependent \(O_2^-\) formation in cell lysates, the cytochrome c reduction method was used as described.\(^ {25,27}\) All of the \(O_2^-\) measurements were performed under NO-synthase inhibition using N\(^\text{N}\)-nitro-L-arginine (30 \(\mu\)mol/L).

**2,7-Dihydrodichlorofluoresceine Fluorescence**

The intracellular formation of ROS was determined by the 2,7-dihydrodichlorofluoresceine (DCF) assay using a confocal microscope (Zeiss LSM 410), as described previously in detail.\(^ {28}\)

**L-012 Chemiluminescence**

\(O_2^-\) formation by PMN induced by phorbol-12-myristate-13-acetate (PMA) was determined using 8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4(2H,3H) dione (L-012).\(^ {26}\)

**Real Time PCR of NAD(P)H Oxidase Membrane Subunits**

Quantitative real-time RT-PCR was performed using commercially available, predeveloped reagents for human Nox1, Nox2, Nox4, and p47\(^\text{phox}\) (TaqMan ABI 7700 Sequence Detection System; Applied Biosystems). The expression of p22\(^\text{phox}\) was determined by semi-quantitative RT-PCR.\(^ {25}\)

**Protein Expression and Rac Pull-Down Assay**

Glutathione S-transferase (GST) alone (control) or GST-PAK1 were cloned, amplified in BCL21 competent cells, and prepared, followed by their use for pull-down assays and blotting using a specific Rac1 antibody (Santa Cruz) as described previously in detail.\(^ {29}\)

**Preparation of Membrane Fractions**

Subcellular fractions of cell membranes were prepared according to the method described by del Pozo et al.\(^ {30}\) Equal amounts of fraction were then analyzed.

**Cell Proliferation and Viability**

Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described before.\(^ {31}\) Cell viability was determined by the trypan blue exclusion method.\(^ {32}\)

**Materials**

Gp91ds-tat and scrambled-tat were kindly provided by Dr Patrick Pagano (Detroit, MI). Superoxide dismutase and dispase were from Roche, endothelial growth medium was from PromoCell, and 2,7-dihydrodichlorofluoresceine was from Molecular Probes. MPA and CsA were kindly provided by Novartis GmbH. CsA by Roche GmbH, and FK506 by Fujisawa GmbH. BCL21-competent cells were from Invitrogen. All of the other substances were from Sigma.

**Statistical Analysis**

For descriptive purposes, all of the data are expressed as mean±SEM. Statistical comparisons with and without treatment within the same experimental group were performed using Student’s \(t\) test or the Wilcoxon signed rank test for paired observations as appropriate. Differences were considered significant when the error probability level was \(P<0.05\).

**Results**

**Calcineurin Inhibitors Increase Endothelial NAD(P)H Oxidase Activity**

CsA and FK506 (0.1 \(\mu\)mol/L each, 24 hours of incubation) increased endothelial \(O_2^-\) production as assessed by 2 independent methods, the cytochrome c (Figure 1) and the DCF fluorescence method (Table), whereas vehicle alone had no effect. The effects of both calcineurin inhibitors were significantly attenuated by the flavoprotein inhibitor diphenyleneiodonium chloride ([DPI] 30 \(\mu\)mol/L; \(n=8\)) or by the specific NAD(P)H oxidase blocker gp91ds-tat (100 \(\mu\)mol/L; \(n=8\)), suggesting an involvement of endothelial NAD(P)H oxidase (Figure 1). A scrambled peptide had no effect (data not shown). Accordingly, NAD(P)H oxidase activity was signif-
significantly increased in lysates obtained from HUVECs treated with CsA or FK506 (0.1 μmol/L each; n=17; 24-hour incubation) from 0.98±0.3 nmol of O$_2^-$ per milligram of protein per minute under control conditions to 3.8±1.0 nmol of O$_2^-$ per milligram of protein per minute (CsA) and 2.8±0.8 nmol of O$_2^-$ per milligram of protein per minute (FK506; $P<0.05$ each; n=17). The viability or proliferation rates of cells remained unaffected by the calcineurin inhibitors (both sets of data are not shown).

**Rapamycin Does Not Alter Endothelial O$_2^-$ Release**

In our experiments in HUVECs, the mammalian target of rapamycin (mTOR) inhibitor rapamycin did not alter endothelial O$_2^-$ release as assessed by cytochrome C assay. Three different doses of the drug (10 nmol/L, 100 nmol/L, and 1 μmol/L), incubated with HUVECs for 2 different time points, did not significantly alter cytochrome C reduction (n=28 to 30; results of 1 μmol/L are shown in Figure 1).

**MPA Inhibits O$_2^-$ Formation by Endothelial NAD(P)H Oxidase**

In contrast to the effects of CsA or FK506, incubation with the IMPDH blocker MPA (1 to 10 μmol/L) markedly attenuated endothelial O$_2^-$ formation (Figure 2A and Table). Although DPI (30 μmol/L) and gp91ds-tat (100 μmol/L) were able to significantly reduce basal HUVEC O$_2^-$ release by 19.8±7% (n=6; $P<0.05$) and 23.0±6% (n=16; $P<0.01$), respectively, they did not affect O$_2^-$ formation after an incubation with MPA any more (5.1±13% and 8.4±7% versus MPA 10 μmol/L; n=8; $P$ value not significant), suggesting that MPA had already blocked this basal activity of endothelial NAD(P)H oxidase. Pretreatment with MPA (10 μmol/L for 24 hours) also significantly attenuated stimulated O$_2^-$ formation in HUVECs, as induced by the protein kinase C (PKC) activator PMA (Figure 2B; PMA 1 μmol/L; n=8). This attenuation was also not affected by gp91ds-tat (100 μmol/L; n=8; Figure 2B). MPA (10 μmol/L) did not alter the reduced nicotinamide-adenine dinucleotide–dependent O$_2^-$ formation in cell lysates (data not shown). Cell viability and cell proliferation were not altered by 24-hour incubation with MPA (data not shown).

**MPA Inhibits Neutrophil NADPH Oxidase**

We further studied whether incubation of neutrophils with MPA also inhibits the respiratory burst induced by the neutrophil NADPH oxidase. As shown in Figure 3, treatment with MPA (10 μmol/L; n=12) for 6 hours also attenuated the PKC-mediated (by PMA; 1 μmol/L) O$_2^-$ formation in neutrophils ($P<0.01$ versus control and $P<0.05$ versus PMA). Acute incubation of PMN with MPA did not affect the signal, indicating that MPA did not act as an O$_2^-$ scavenger.

**MPA Does Not Alter Transcriptional NADPH Oxidase Activation**

To investigate whether the decrease in O$_2^-$ formation was because of transcriptional inactivation of NAD(P)H oxidase, we performed semiquantitative RT-PCR and real-time RT-PCR experiments on mRNA expression of several NAD(P)H oxidase membrane subunits. The expression of the p22phox*, as assessed by semiquantitative RT-PCR, remained unaltered by...
MPA (Figure 4A; n = 5). Using real-time RT-PCR endothelial expression of Nox2, Nox4, and p47^phox also did not show altered expression after treatment with MPA (Figure 4B; n = 6). Expression of Nox1 was not detected by RT-PCR in these cells. Normalization to 2 different housekeeper genes (GAPDH and 18S rRNA) yielded comparable results.

MPA Attenuates Endothelial Activation of the Small GTPase Rac1
To further study the mechanism underlying inhibition of endothelial NAD(P)H oxidase activation by MPA, we next examined its effects on the activation of the small GTP-binding protein (GTPase) Rac1, which we have observed recently to be activated during endothelial NAD(P)H oxidase activation.27 MPA (10 μmol/L; n = 7; 24-hour incubation) not only reduced the amount of membrane-bound Rac1 (Figure 5A), but also led to a marked attenuation of Rac1 activation as indicated by binding of activated Rac1 to GST-PAK in a specific pull-down assay (Figure 5B; n = 4; mean band density ratios for controls 0.88±0.17 and for MPA 0.42±0.34; P = 0.054). Restoration of cellular GTP-content by addition of guanosine (1 μmol/L; n = 6) abolished this inhibitory effect of MPA (10 μmol/L), as suggested by measurements of endothelial O_2^- formation by the use of 2 independent methods (Figure 6 and Table 1; results obtained by the cytochrome c method are not shown).

Discussion
In this study, we highlight an important difference between immunosuppressive agents that are currently used for prevention of cardiac allograft rejection. Although the calcineurin...
inhibitors CsA and FK506 may deteriorate endothelial cell function by significantly increasing the activity of NAD(P)H oxidase and subsequent $O_2^-$ formation at clinically relevant doses, the IMPDH blocker MPA inhibits this enzyme. Moreover, by showing that MPA leads to an inactivation of the NAD(P)H oxidase subunit Rac1, we unveil the mechanism by which MPA exerts its inhibitory action on endothelial $O_2^-$ formation. We did not find an effect of the mTOR inhibitor rapamycin on endothelial $O_2^-$ release in this study.

It has been shown previously that CsA increases vascular ROS synthesis, but the mechanisms and enzymatic sources remained unclear.15 Our data, for the first time, indicate that not only CsA, but also the more potent calcineurin inhibitor FK506, increase endothelial NAD(P)H oxidase activity, as demonstrated by use of the highly specific inhibitor of NAD(P)H oxidase gp91ds-tat and direct assessment of the enzyme activity. Although the signal pathways underlying these processes were not the

**Figure 4.** MPA does not alter the expression of NAD(P)H oxidase subunits. A, p22phox as measured by RT-PCR. HUVECs were incubated with MPA (10 μmol/L) for 24 hours, and mRNA expression of the subunit p22phox was determined. A typical blot from 5 independent experiments is shown. B, Real-time RT-PCR of different Nox subunits and of p47phox. mRNA expression of Nox2, Nox4, and p47phox was not influenced by MPA (n=6; P value not significant); Nox1 mRNA was not detected (data not shown).

**Figure 5.** MPA inhibits activation of Rac1: (A) incubation of HUVEC with MPA (10 μmol/L) reduced the amount of membrane bound Rac1. A representative blot of 7 independent experiments is shown (*P<0.05 vs control). B, The activity of the small GTPase Rac1 was measured using a specific Rac1 pull-down assay as described above. When HUVECs had been incubated for 24 hours with MPA (10 μmol/L), the amount of activated Rac1 (GTP-bound Rac1) was markedly reduced. The Western blot analysis shows pull-down samples (activated Rac1) at the top as compared with total Rac1 protein (bottom). The GTP-Rac1 band densities to total Rac1 band density ratio showed a strong trend toward significance (bottom; n=4; P=0.054).
subject of this study, it can be speculated that an activation of calcium-dependent isoforms of PKC by calcineurin inhibitors could have been involved in this NAD(P)H oxidase activation.35 Our results support the view that calcineurin inhibitors decrease availability of endothelial NO and impair endothelial function.

In contrast, MPA had an inhibitory effect on endothelial O$_2^-$ generation. Our data suggest that MPA reduced endothelial ROS formation by blockade of the constitutively active endothelial NAD(P)H oxidase, because both NAD(P)H oxidase inhibitors that were used failed to have an effect on O$_2^-$ release after preincubation with MPA, which itself significantly reduced basal O$_2^-$ formation. This was neither because of scavenging of O$_2^-$, as addition of MPA had no immediate effect on O$_2^-$ levels, nor because of transcriptional alteration of NAD(P)H oxidase, as endothelial mRNA expression of several subunits of NAD(P)H oxidase remained unaltered. These include the large electron transferring subunit Nox4, which has been reported to be detected only in cells of the immune system.40 This suggests that the activation of Rac1, which in contrast to neutrophils, where Rac2 is expressed, is the endothelial Rac isoform participating in NAD(P)H-oxidase activation and may have been inactivated. Accordingly, we observed that incubation of HUVECs with MPA reduced the amount of membrane-bound Rac1, as well as its activity. These results further suggested that MPA-induced inhibition of endothelial O$_2^-$ formation was because of NAD(P)H oxidase inactivation, because Rac1 is not only an essential component of endothelial.25,27 NAD(P)H oxidase, but of several vascular NAD(P)H oxidases isoforms.36,37 Indeed, there have been reports about MPA (10 $\mu$mol/L) reducing cellular GTP content in HUVECs.38 However, direct measurement of GTP levels is hampered by the low in vitro availability of this nucleotide; therefore, most studies rely on indirect proof by restoring GTP levels through exogenous addition of guanosine as source for IMPDH independent GTP synthesis, which prevented the inhibitory effect of MPA on proliferation of cultured mesangial as well as smooth muscle cells in previous studies.4,39–41 In the present study, supplementation of guanosine similarly restored the effects on Rac1 activity and O$_2^-$ formation in our assays, confirming previous studies that decreased availability of GTP may be associated with impaired function of small GTP-binding proteins.18

In summary, the present study demonstrates that MPA inhibits endothelial O$_2^-$ formation by inhibition of the NAD(P)H oxidase, whereas the calcineurin inhibitors CsA and FK506 increased its activity. We speculate that this effect of MPA was caused by depletion of cellular GTP content, which was the likely cause for subsequent attenuation of Rac1 activity. Although mTOR inhibitors, such as rapamycin or everolimus, have been observed to have beneficial effects on CAV in clinical settings as well,42,43 we did not observe an effect of rapamycin on endothelial O$_2^-$ release in vitro.

**Perspectives**

Because endothelial dysfunction precedes the development of CAV and cardiovascular events,9 and recent clinical studies support the notion that MPA may be beneficial in reducing the risk of CAV,44 immunosuppressive regimen that include MPA should be considered to maintain endothelial function and, thus, prevent development of CAV or hypertension. Moreover, because oxidative stress is...
equally involved in the development of hypertension in other organ transplant recipients, such as kidney transplant patients, our findings may also be of importance for the prognosis of these patients.45

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Disclosures
None.

References


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