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# Vascular targets of redox signalling in diabetes mellitus

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## Abstract

There is overwhelming evidence for an involvement of reactive oxygen species (ROS) in the pathogenesis of diabetes-associated vascular complications. However, neither the exact source of the ROS initiating cascades leading to cell dysfunction in diabetes nor their chemical nature is fully understood. Furthermore, despite our knowledge of the crucial role of ROS in diabetes, little is known about the actual targets and the molecular consequences of the interaction of ROS with cellular signalling pathways.

Therefore, we aim to provide an overview of ROS (i. e. $O_2^{\bullet-}$, $NO^{\bullet}$, $ONOO^-$ and $H_2O_2$) and their vascular sources in diabetes and to summarise recent knowledge on the mechanisms underlying increased ROS production within the vascular wall. In addition, possible targets of diabetes and ROS within the vasculature are discussed. These include, the effects of ROS on small guanine nucleotide binding proteins, the cytoskeleton, protein kinases (e. g. tyrosine kinases), metalloproteinases, ion homeostasis and transcriptional regulation.

Such analysis makes it clear that the generation of ROS could affect a large number of various signalling pathways and proteins. Thus, a better knowledge of the functional diversity and pathological consequences of each individual pathway activated by ROS is essential to understand the mechanisms of diabetes-associated vascular complications. [Diabetologia (2002) 45:476–494]

**Keywords** Diabetes, reactive oxygen species, superoxide, nitric oxide, RhoA, Rac, tyrosine kinases, serine/threonine kinases, $Ca^{2+}$-homeostasis, NF-$\varkappa$B, NFAT, AP-1, matrix metalloproteinases.

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*Abbreviations:* AA, Arachidonic acid; AGE, advanced glycation end product; AngII, angiotensin II; AP-1, activator protein-1; ATF-1/2, Activated transcription factor-1/2; $BH_4$, tetrahydrobiopterin; BHA, 2(3)-t- Butyl-4-hydroxyanisole; BMK1, big MAPK; t-BuOOH, tertiary Butylhydroperoxide; Cas, crk-associated substrate; *CDC42,* homologous to yeast cell division cycle gene 42; CHOP, C/EBP homologous protein (stress activated transcription factor); COX, cyclooxygenase; CREB, cAMP-responsive element binding protein; Crk p130, CT-10 regulated kinase; CYP450, cytochrome P450 monooxygenase; DPI, diphenylene iodonium; EC, endothelial cell; EDHF, endothelial derived hyperpolarising factor; ERK 1/2, extracellular signal-regulated kinase 1/2; FAK, focal adhesion kinase; $H_2O_2$, hydrogen peroxide; HMG, hydroxymethylglutaryl; HX, hypoxanthine; $IP_3$(R), Inositol- 1, 4, 5- triphosphate (receptor); JNK, cJun N-terminal kinase; LDL, low density lipoprotein; L-NNA, L-nitroarginine; $LO^{\bullet}$, lipid alkoxyl radical; $LOO^{\bullet}$, lipid peroxyl radical; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MEF2 (RSRF), myocyte enhancer factor2 (serum response factor-related proteins); MMP, matrix metalloproteinase; MPO, myeloperoxidase; NAC, N-acetyl-L-cysteine; NF-AT, nuclear factor of activated T cells; *NF-*$\varkappa$B, nuclear factor-$\varkappa$B; NGA, nordihydroguaiaretic acid; NHE, $Na^+/H^+$ exchanger; NOS, NO synthase; eNOS, endothelial NOS; NAD(P)H, oxidase; $O_2^{\bullet-}$, superoxide anions; $ONOO^-$, peroxynitrite; Phox, phagocytic oxidase; PI3-kinase, phosphatidylinositol 3 kinase; PKB, protein kinase B (Akt); pS, picosiemens; Rac, ras-related $C_3$-botulinum toxin substrate; Ras, rat sarcoma virus; Rho, ras homologous; ROS, reactive oxygen species; p90RSK, 90 kDa ribosomal S6 kinases; = MAPKAP-K1, MAPK-activated protein kinase-1; RyR, ryanodine receptor; SAPK, stress-activated protein kinase; SERCA, $Ca^{2+}$ATP-ase; SMC, smooth muscle cell; SOD, superoxide dismutase; TCF (Elk-1), ternary complex factor; TK, tyrosine kinase; TNF, tumour necrosis factor; TPA, 12-O- tetradecanoylphorbol-13-acetate; TRE, TPA responsive element; TRP, transient receptor potential; X, xanthine; XOX, xanthine oxidase

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes mellitus. Because the onset and progression of associated complications are delayed in patients with good glycaemic control, hyperglycaemia appears an important regulator of vascular lesion development. Diabetes and hyperglycaemia are strongly associated with endothelial dysfunction, which is an early hallmark in the development of atherosclerosis. The term endothelial dysfunction has been used for several pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, dysregulation of vascular remodelling and impaired endothelium-dependent vasorelaxation caused by the loss of nitric oxide ($NO^{\bullet}$) bioactivity in the vessel wall.

Furthermore, alteration of platelet function contributes to microthrombus formation and could play an important role in the pathogenesis of diabetic micro and macroangiopathies. Hyperglycaemia potentiates platelet aggregation, and its subsequent release of platelet-derived growth factor, AB, inhibits protein tyrosine phosphatase (PTP) activity and increases phosphorylation of tyrosine kinases in platelets exposed to collagen. Inhibition of the respiratory chain or application of SOD-mimetic prevents these effects, indicating that mitochondrial superoxide generation could play an important role in platelet dysfunction observed in patients suffering from diabetes [1].

Recent studies indicate that higher D-glucose concentrations change several intracellular signal transduction cascades in the vascular wall, including modulation of protein kinase C (PKC) (for review see [2]), activation of mitogen-activated protein kinases and the generation of reactive oxygen species (ROS). Furthermore, hyperglycaemia-induced oxidative stress might be the cause as well as the result of the accumulation of advanced glycation end products (AGEs) or both [3] that have been demonstrated to enhance the expression of vascular cell adhesion molecule-1 and oxidative stress upon interaction with specific receptors [4].

While the ultimate effects of diabetes and hyperglycaemia on blood vessel function is well documented, the underlying mechanisms leading to dysfunctions are not clear. During the last few years, convincing evidence indicates that the generation of ROS plays a crucial role in the development and the progression of vascular dysfunction associated with a variety of diseases, such as hypercholesterolaemia [5, 6, 7], hypertension [8, 9] and diabetes mellitus [1, 8, 10, 11]. Under these conditions an excessive endogenous formation of ROS apparently overcomes cellular antioxidant defence mechanisms, resulting in ROS-initiated modification of lipids, proteins, carbohydrates and DNA [12]. This condition is commonly termed oxidative stress. In addition to the oxidative modification of target molecules affecting their function, distribution or metabolism, ROS, such as superoxide anions ($O_2^{\bullet-}$) or nitric oxide ($NO^{\bullet}$) also affect or initiate signal cascades directly. Remarkably, $O_2^{\bullet-}$ seems to function as a messenger in signal transduction that mediates downstream signalling (e.g. Ras and Rac) even under physiological conditions [13]. Although in mammalian cells there is no proof for proteins capable of sensing $O_2^{\bullet-}$ or $H_2O_2$, growing evidence supports the concept that $O_2^{\bullet-}$ and probably other oxidants serve as physiological signal molecules at lower concentrations, while larger amounts of the same ROS induce cell damage and dysfunction [14].

Thus, the production and action of ROS represent crucial phenomena involved in physiological and pathological mechanisms. This review aims to provide an overview of ROS-sensitive signalling proteins and pathways that forward a signal to alter cell function and gene expression. This review describes proteins and signal cascades that contribute to the development of diabetes-associated vascular dysfunction and blood vessel complications, but does not claim completeness.

## Chemistry of ROS

The term ROS describes a group of small, reactive oxygen-containing molecules that are either free radicals containing oxygen or nitrogen-based unpaired electrons or compounds that are not free radicals themselves, but have oxidising properties that contribute to oxidant stress (Fig. 1).

Among the free radicals, superoxide anions ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), nitric oxide ($NO^{\bullet}$) and lipid radicals ($LO^{\bullet}$, $LOO^{\bullet}$) are the most prominent. Remarkably, not all of these oxygen-containing radicals have high oxidative potential and reactivity to interact with cellular molecules such as proteins or lipids. Thus, the half-life of these radicals in vivo varies from few milliseconds (e.g. $HO^{\bullet}$) up to several seconds and even minutes (e.g. $O_2^{\bullet-}$, $LOO^{\bullet}$). Non-radical ROS, such as hydrogen peroxide ($H_2O_2$), hypochloric acid (HOCl) and peroxynitrite ($ONOO^{-}$), are compounds that emerge under oxidative stress and mediate oxidative signalling to their environment. $ONOO^{-}$ is formed by the ultrafast reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$, while HOCl and $H_2O_2$ are products of the myeloperoxidase (MPO) and superoxide dismutase (SOD), respectively. $O_2^{\bullet-}$ are probably not the only radicals that react with $NO^{\bullet}$. Lipid radicals ($LO^{\bullet}$ and $LOO^{\bullet}$) can react with $NO^{\bullet}$ to form LONO and LOONO [15]. Recently it has been shown that even a reaction between $HO^{\bullet}$ and $NO^{\bullet}$ is possible [16].

It is obvious that ROS differ considerably in terms of the effects they mediate in intact cells. Thus, in or-

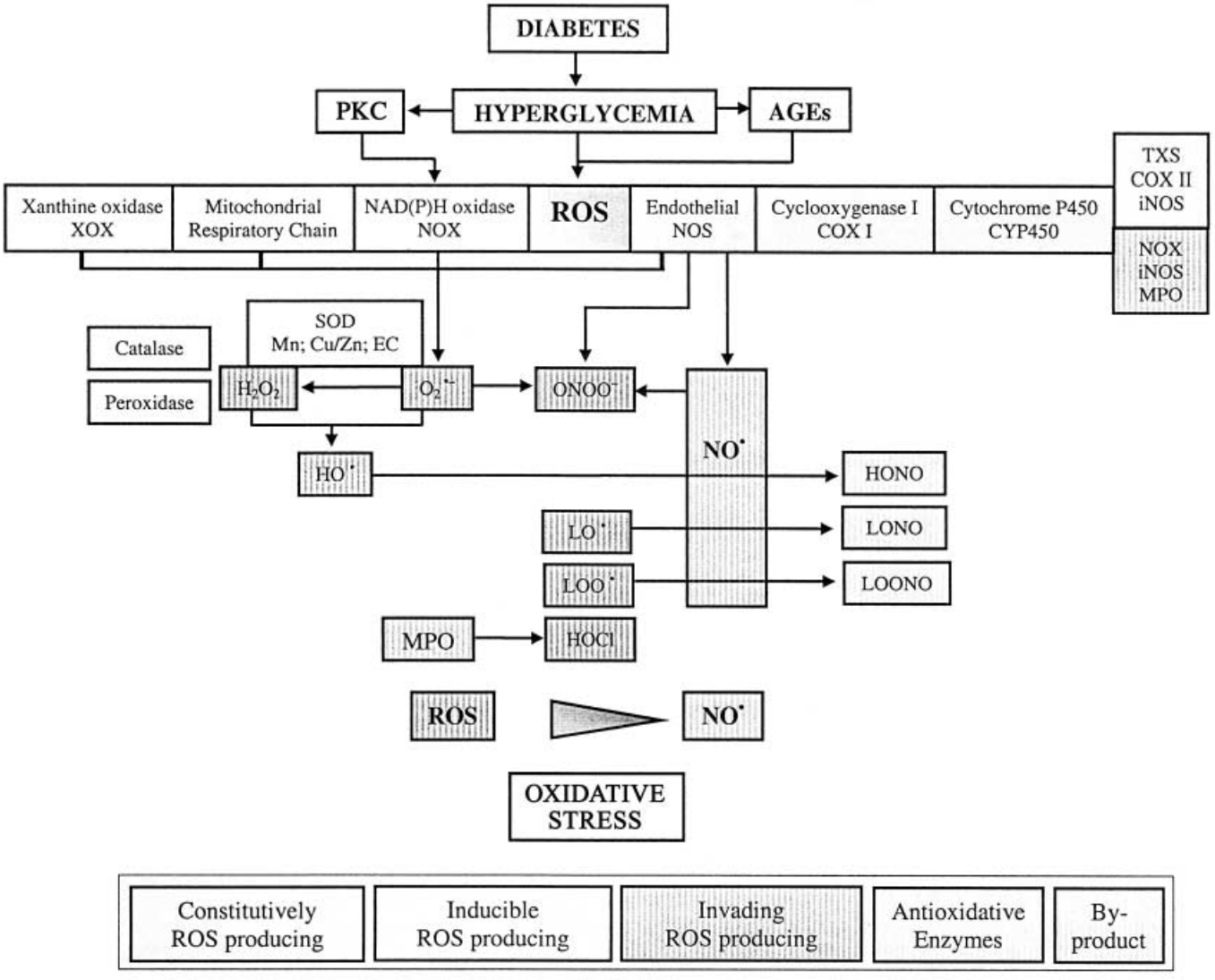


**Fig. 1.** Major ROS and their sources involved in diabetics processes

der to assess the effect of ROS on the vasculature, each individual reactive oxygen molecule needs to be performed separately.

## ROS in the vascular wall

Several sources of ROS (Fig. 1) contribute to the development of circulatory complications and vascular dysfunction. Enzymes responsible for ROS formation in the vascular wall can be divided into three groups: enzymes that are (i) constitutively active in the vascular wall, (ii) that are induced in vascular cells under pathological conditions, and (iii) ROS producing enzymes that are imported into the vascular wall by invading cells. Members of the first group are cyclooxygenase (COX I), lipooxygenase [17], cytochrome P450 (CYP450), xanthine oxidase (XO), NAD(P)H oxidase, superoxide dismutase (SOD), the endothelial nitric oxide synthase (eNOS) and the enzyme complexes contributing to the mitochondrial respiratory chain. While the expression of these constitutive enzymes is enhanced in various diseases, the expression of inducible enzymes, such as thromboxane synthase (TXS), cyclooxygenase 2 (COX II) and inducible nitric oxide synthase (iNOS) is switched on under pathological conditions. Monocyte NADH/NADPH oxidase, monocyte and macrophage iNOS (iNOS) and myeloperoxidase (MPO) [18] belong to the group of ROS-producing enzymes that are imported by invasion of blood cells into the vascular wall.

$O_2^{\bullet-}$ and $H_2O_2$ can interact with each other or with iron or copper containing molecules (Fenton- or Haber-Weiss- reaction) to generate the highly reactive HO$^\bullet$. In addition to enzymatically produced ROS, the cellular production of one ROS can lead to the production of several others through radical chain reactions. The reaction between radicals and

polyunsaturated fatty acids within cell membranes results in a fatty acid peroxyl radical (R-COO$^{\bullet}$) that, in turn, attacks adjacent fatty acid side chains to produce other lipid radicals. These accumulate in the cell membrane and can have multiple effects on cellular function, including plasma membrane leakage and dysfunction of membrane bound receptors [8]. Finally, the generation of ROS also occurs as a result of non-enzymatic processes that involve the autoxidation of D-glucose in the presence of transition ions such as $Fe^{2+}$ or $Cu^{+}$ [19, 20]. Notably, transition ions that could serve as a source of metal-catalysed ROS can be sequestered by glycated proteins [21, 22].

There are five enzymes or enzyme-complexes that are frequently discussed to contribute to diabetes-associated ROS generation within the vascular wall (Fig. 1).

*NAD(P)H oxidase.* Several isoforms of this multienzyme complex have been found in the vascular wall [9]. In general, enzyme activity is provided by the cytochrome $b_{558}$, an integral membrane protein composed of the gp91phox and p22phox subunits. The activity of $b_{558}$ is dependent upon its interaction with the additional components p67phox, p47phox, p40phox and the small G protein, ras-related $C_3$-botulinum toxin substrate (Rac) [14, 23]. The two isoforms of Rac, Rac1 and Rac2 [24, 25], promote the assembly of the NAD(P)H oxidase [25, 26] as well as the stability of this multicomplex enzyme [25, 26]. While Rac2, which has a higher affinity for the NAD(P)H oxidase than Rac1 [14], seems to be constitutively associated with membranes, Rac1 translocates from the cytosol to the membrane together with the other components upon stimulation of the respiratory burst. Subsequently, in experimental hyperglycaemia, the production of $O_2^{\bullet-}$ by endothelial and smooth muscle NAD(P)H oxidase has been discussed [27, 28, 29]. In line with the potential role of NAD(P)H oxidase in the diabetic vessel, NADH-dependent generation of $O_2^{\bullet-}$ was described in endothelium and vascular smooth muscle cells [30, 31, 32, 33]. In segments of human saphenous veins obtained from patients undergoing routine coronary artery bypass surgery [34] and in uterine arteries from diabetic patients increased NAD(P)H oxidase-dependent $O_2^{\bullet-}$ production occurs. In agreement with these findings, Inoguchi and co-workers reported the PKC-mediated formation of $O_2^{\bullet-}$ in cultured vascular cells under hyperglycaemic conditions which was sensitive to diphenylene iodonium (DPI), an inhibitor of flavoproteins (e. g. NAD(P)H oxidase, eNOS) [29]. The involvement of PKC in diabetes-mediated vascular dysfunction (for review see [2]) has been shown in animal models [35, 36]. Moreover, PKC activation is involved in the regulation of eNOS and endothelin-1 expression under diabetic conditions [37, 38]. Thus, although PKC might activate NAD(P)H oxidase in diabetes, the underlying mechanisms [24, 25, 26, 39] need further investigation.

*Thromboxane synthase.* The thromboxane synthase produces prostaglandin endoperoxides in a cage radical mechanism [40]. Interestingly, in the aortas of diabetic rabbits impaired endothelium-dependent relaxation is associated with increased production of thromboxane $A_2$ or its precursor prostanoid, prostaglandin endoperoxide (PGH; [41]). Further evidence for the involvement of thromboxane synthase in diabetes-associated vascular complications comes from the findings that inhibitors of the thromboxane synthase prevented renal injury [42] and retinopathy [43, 44] in diabetic animals. In humans, inhibitors of the thromboxane synthase were described as being beneficial against diabetic nephropathy [45]. Furthermore, in the aortae of diabetic rats the selective inhibitor of thromboxane $A_2$-prostaglandin/$H_2$ receptors, ONO-3708, abolished acetylcholine-induced contraction and prevented the diabetes-associated impairment of endothelium-dependent blood vessel relaxation ([46]). In agreement with these results endothelial thromboxane $A_2$ formation was reported under hyperglycaemic conditions [47, 48].

*Superoxide dismutase (SOD).* SOD represents one of the major ROS-defending enzymes. However, it is noteworthy that during the SOD-mediated conversion of the $O_2^{\bullet-}$ to $H_2O_2$ a further ROS is produced. These two ROS differ greatly in terms of their physical properties and biological action. $O_2^{\bullet-}$ cannot freely penetrate the cell membrane and would need to pass through certain ion channels ($Cl^-$ channels) [49, 50], while $H_2O_2$ can easily enter through the cell membrane. Thus, the extracellular conversion of $O_2^{\bullet-}$ to $H_2O_2$ by extracellular SOD, that is bound on endothelial surface matrix by heparansulfate residues, might generate a membrane penetrating molecule that transduces extracellular oxidative stress into the cells. While this aspect has been rarely discussed, SOD was widely used to prevent hyperglycaemic/diabetic vascular dysfunction [11, 51, 52, 53, 54, 55]. Importantly, most of these studies were focussed on acute hyperglycaemia, endothelial reactivity or apoptosis [53], whereas the effect of SOD on vascular adaptation and changes in response to diabetic conditions has not been investigated so far. Because SOD and catalase are able to restore completely the dilatory response to acetylcholine in diabetic animals, it is tempting to speculate on an involvement of $O_2^{\bullet-}$ and $H_2O_2$ in the impairment of endothelium dependent dilation in diabetes [55, 56].

While hyperglycaemia changes endothelial SOD expression [57], the expression of SOD in diabetic arteries was found to be either unchanged, increased or decreased depending on disease duration [58, 59, 60]. Nevertheless, the importance of SOD as a defence

protein against diabetes-associated reduction of blood vessel relaxation is apparent from the exciting finding that gene transfer of CuZnSOD and MnSOD reversed endothelial dysfunction aortae of diabetic rabbits [61]. Thus, considering the possible alterations in SOD expression, its possible beneficial effects on diabetic endothelial dysfunction and the different effects of its substrate $O_2^{\bullet-}$ and the resulting product $H_2O_2$, the role of SOD in vascular oxidative defence and in the development of vascular dysfunction needs to be explored in more detail.

*Endothelial nitric oxide synthase (eNOS).* Despite attenuation of $NO^\bullet$ production [62] and its bioactivity [11, 63] by high glucose concentrations, the expression of endothelial nitric oxide synthase (eNOS, NOS III) has been found to be upregulated in endothelial cells under such conditions [64]. In line with these findings, increased eNOS expression was found in the aortae of streptozotocin diabetic rats [10], while in the same model a reduced eNOS protein content was found in the skeletal muscle [65] and in the heart [66]. No change in eNOS expression was seen in diabetic rats (BB/W) [67]. There is evidence that the reduced $NO^\bullet$ production by eNOS in diabetes [68] might be due to its substrate L-arginine [53, 69], a posttranslational modification of the eNOS on the Akt phosphorylation site [71] and the lack of its coenzyme tetrahydrobiopterin [53, 70]. The latter results from decreased expression and, activity of GTP-cyclohydrolase I, the first and rate limiting enzyme in the de novo biosynthesis of $BH_4$, in diabetic cells [72]. In studies on the purified enzyme the lack of either tetrahydrobiopterin or L-arginine or both results in eNOS uncoupling [73, 74] which is indicated by a $Ca^{2+}$-dependent formation of $O_2^{\bullet-}$ [75]. It has been suggested that $ONOO^-$ oxidises $BH_4$, which can uncouple eNOS in vivo [76] and thereby contributes in the endothelium to oxidative stress and endothelial dysfunction through at least 3 mechanisms. First, the enzymatic production of $NO^\bullet$ is diminished, thus the system lacks $NO^\bullet$ as an essential mediator molecule. Second, the enzyme produces $O_2^{\bullet-}$ and thus, contributes to oxidative stress. Finally, it is likely that eNOS becomes partly uncoupled, so that both $O_2^{\bullet-}$ and $NO^\bullet$ are produced simultaneously. Under these circumstances, eNOS could become a $ONOO^-$ generator, leading to a dramatic increase in oxidative stress. Recently, the production of $O_2^{\bullet-}$ by eNOS has been demonstrated in streptozotocin-diabetic rats [10]. According to this report eNOS-mediated $O_2^{\bullet-}$ production in diabetes critically depends on PKC activation which supports the findings of PKC activation as a crucial step in the development of diabetic vascular complications [35, 77–79].

A decline in $NO^\bullet$ bioavailability could be caused by reduced expression of eNOS [80], a lack of substrate or cofactors of eNOS [73], increased $NO^\bullet$ scavenging by $O_2^{\bullet-}$ [81, 82] and alterations of cellular signalling so that eNOS is not appropriately active [55, 83].

*Mitochondria.* Pathological changes caused by diabetes are consequences of hyperglycaemia-induced mitochondrial superoxide overproduction [78, 79]. Under hyperglycaemic conditions, mitochondrial $O_2^{\bullet-}$ has been shown to activate sorbitol accumulation by the aldose-reductase pathway [84] resulting in the activation of PKC [77, 78] which, in turn, is responsible for the generation of AGEs and endothelial dysfunction. The metabolism of D-glucose through the sorbitol pathway has been reported to affect the oxidative potential of endothelial cells [85, 86]. This hypothesis suggests that an enhanced catabolism of D-glucose through the sorbitol pathway yields a status of pseudohypoxia (increased NADH concentrations), activates PKC [84] and subsequent downstream signalling. In addition, mitochondrial superoxide overproduction inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity and activates the hexosamine pathway, presumably by diverting the upstream metabolite fructose-6-phosphate from glycolysis to glucosamine formation [87]. In heart failure it has been shown that mitochondria complex I is the predominant source of the primary radical $O_2^{\bullet-}$ [88] which, in turn, is converted to $H_2O_2$ and the potent reactive species $HO^\bullet$ [89].

In addition to the role of mitochondria as a source of ROS, the mitochondria themselves can be damaged by oxidants. ROS mediate mitochondrial DNA damage, alterations in gene expression and mitochondrial dysfunction in cultured vascular ECs and SMCs [90].

## Targets of ROS

*Small guanine nucleotide binding proteins.* Small guanine nucleotide binding proteins (G-proteins) exert GTPase activity and are proposed to serve as mediators of ROS (Fig. 2). The Rho GTPase family is a member of the Ras superfamily [91] and consists of at least 14 distinct proteins ranging from 20 to 24 kDa, which can be additionally subdivided. Small G-proteins of the Rho subfamily, such as Rho, Rac and CDC42 are involved in many cellular processes, including proliferation, differentiation [92], migration, cytoskeletal organisation and signal transduction [93, 94] and might play a crucial role in the development of cardiovascular complications. In certain cell types, Rho, which probably mediates ROS production, triggers the activation of the proinflammatory nuclear transcription factor (NF-$\varkappa$B) in response to hyperglycaemia and AGEs [95]. While Rac has been shown to constitute a mediator of the oxidant-initiated signalling [96, 97], it is also a target for $H_2O_2$,

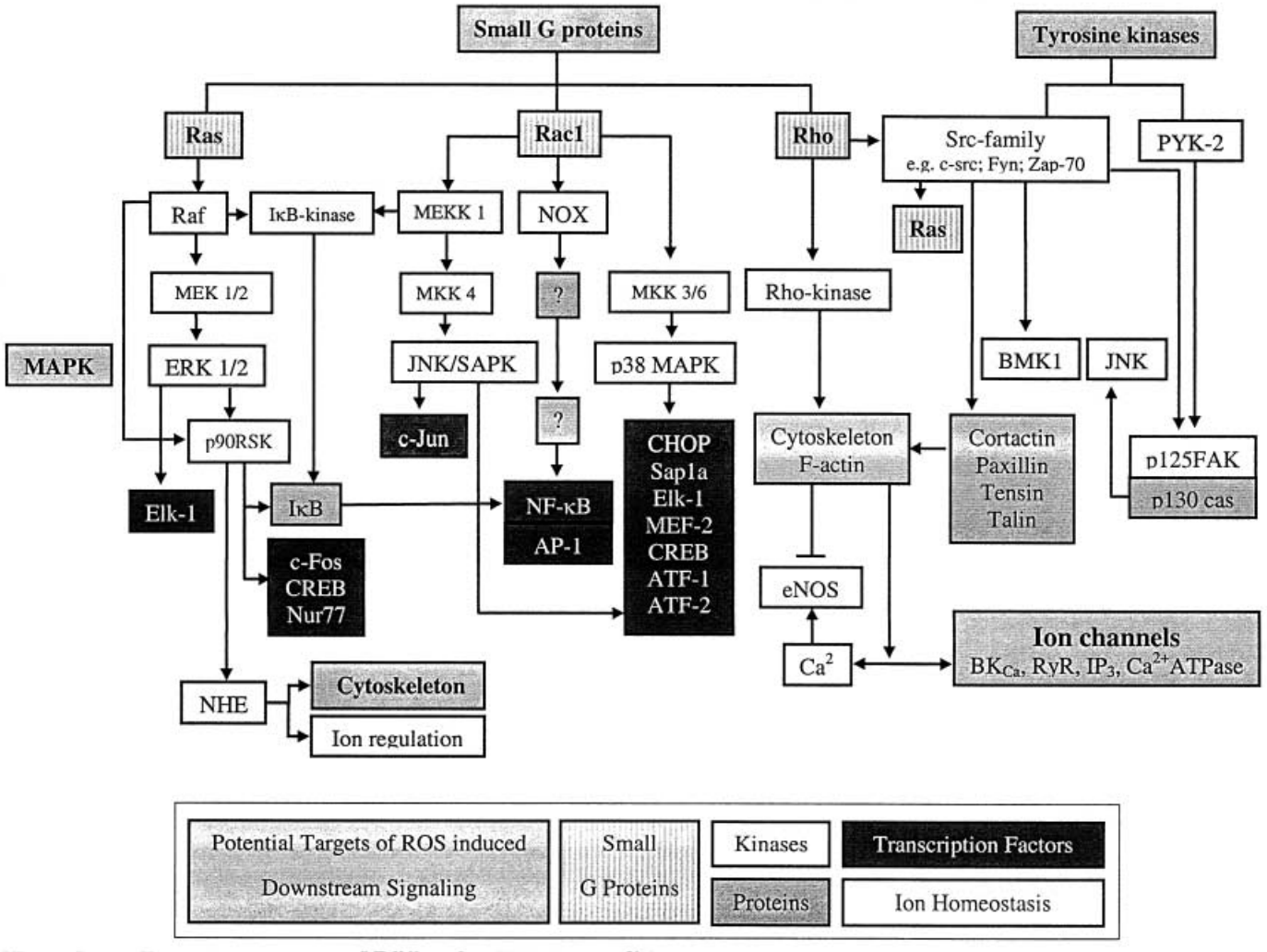


**Fig. 2.** Downstream targets of ROS under diabetic condition

$NO^{\bullet}$ and $O_2^{\bullet-}$ [13]. In addition, ROS could directly activate Ras that, in turn, initiates activation of the mitogen-activated protein kinase (MAPK) pathway [13]. The sites of oxidative attack of H-Ras are the cysteine residues contributing to membrane binding and Cys-118. Modification of the latter affects the binding of GTP and GDP on H-Ras [98, 99]. The Rho family member Rac1 is a regulatory component of the NAD(P)H oxidase (see above) in many cell types, including neutrophils and cells of the vascular wall. Rac triggers the clustering of the subunits of NAD(P)H oxidase to form the functional multienzyme complex [14].

These cytosolic GTP-binding proteins are isoprenylated and some are additionally palmitoylated on cysteine residues [100], allowing translocation to the cell membrane as the initial step in their activation. The beneficial effects of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) which block geranylgeraniol synthesis in hypertension and heart failure, could be because of the inhibition of Rho proteins in the heart. Indeed, inhibition of Rac1 isoprenylation by statins inhibits the release of reactive oxygen species in endothelial cells [101]. In vascular smooth muscle cells, Rho promotes cell-cycle progression and proliferation, which are central events in the pathogenesis of vascular lesions, including postangioplasty restenosis, transplant arteriosclerosis and vein craft occlusion. The molecular mechanism is attributable, in part, to Rho-induced destabilisation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ [102]. Recent studies also suggest that statins exert additional anti-inflammatory and antioxidant effects on the vascular wall [101, 102].

Furthermore, Rho plays an important role in the regulation of endothelial function and gene expression [103]. Besides upregulating preproendothelin-1 expression, RhoA negatively regulates the production of endothelium-derived $NO^{\bullet}$ by Rho-induced changes in the endothelial actin cytoskeleton [104].

*Small guanine nucleotide binding proteins and the cytoskeleton.* Small G-proteins like RhoA, Rac and CDC42 represent key regulators of the actin cytoske-

leton [105] and induce assembly of stress fibres and focal adhesions (RhoA), membrane ruffling, redistribution of actin fibres and an increase in the total amount of f-actin (Rac) and filopodia formation (CDC42). $O_2^{\bullet-}$ induced changes in the actin cytoskeleton (Fig. 2), such as the polymerisation of f-actin to stress fibres with a concomitant increase in f-actin, are associated with reinforced tyrosine phosphorylation. The actin reorganisation in response to $O_2^{\bullet-}$ is thought to be mediated by changes in the redox state of actin or its regulatory (sequestering or capping) proteins [14, 102]. Alternatively, $O_2^{\bullet-}$ might be involved in cytoskeletal changes by the activation of Rac [14, 106]. In line with this hypothesis, overexpression of Rac in human and mouse aortic ECs induces an increase in f-actin content and actin reorganisation which is accompanied by the $O_2^{\bullet-}$ production [102].

Increasing evidence points to a central role of Rho in the regulation of the actin cytoskeleton by mediating changes in cell shape, contractility and motility. The functional linkage between Rho and the cytoskeleton was further supported by the findings that a direct inhibition of Rho by Clostridium botulinum C3 transferase and the disruption of the endothelial actin cytoskeleton by cytochalasin D increase expression and activity of aortic eNOS [107].

Clinical trials on statins point to a beneficial potential of these drugs in cardiovascular diseases that exceeds their effect on cholesterol-lowering [102]. Because statins also inhibit isoprenoid synthesis [102] which is required for the posttranslational modification of Rho it is tempting to speculate that at least parts of their action are due to direct effects on Rho which is required for basal expression of preproendothelin-1 in vascular endothelial cells. In addition statins inhibit preproendothelin-1 expression by blocking Rho geranylgeranylation [103]. Hence, inhibition of Rho is an important effect of the statins independently to their cholesterol-lowering properties. Therefore, small G proteins like Rac and Rho are likely to be involved in the development of diabetes and ROS-associated vascular complications.

*Protein kinases.* ROS activate several proteins of important signalling cascades that individually change cell function and gene expression.

The activation of PKC represents a hallmark in the development of vascular complications in diabetes mellitus (for review see Idris et al. [2]). Among the PKC family, several isoenzymes have been shown to contribute to insulin signalling (e.g. PKC-$\theta$ and PKC-$\varepsilon$) and insulin-stimulated D-glucose uptake (PKC-$\zeta$ and PKC-$\lambda$), while others (most prominently PKC-$\beta$) are involved in the development of diabetes-linked complications. This review consequently focuses on other potential signalling kinases (Fig. 2; Table 1) that might constitute additional targets in the pathology of diabetes.

Among the growing number of ROS-sensitive signalling cascades, the activation of *serine/threonine protein kinases* such as mitogen-activated protein kinases (MAPKs) or Akt kinase (PKB) have been described. A member of the MAPK family, the extracellular signal-regulated kinase (ERK1/2) was found to be activated by exogenous $H_2O_2$ and endogenously generated ROS [108] by PKC, Raf-1 and MEK1 [109]. Based on these findings, it was hypothesised that reactive oxygen species might activate MAPK in vascular smooth muscle cells as $O_2^{\bullet-}$ but not $H_2O_2$ stimulated activation of MAP kinase, which if due to $O_2^{\bullet-}$ was PKC dependent [13]. On the contrary, Guyton et al. [110] demonstrated ERK activation as well as a moderate stimulation of c-Jun N-terminal kinase (JNK) and p38 MAPK by $H_2O_2$ in several cell types. In line with these findings, an inhibition of p38 MAPK was found to ameliorate diabetes-associated vascular dysfunction in the rat mesenteric microcirculation [111]. Another MAPK, BMK1, a downstream target of c-src, seems to be specifically redox-sensitive. In smooth muscle cells it has been observed that in response to several different agonists BMK1 was stimulated to the greatest extent by $H_2O_2$ with a relative potency of $H_2O_2 > > > PDGF > PMA = TNF\text{-}\alpha$ [112].

Stress-Activated Protein Kinases (SAPK), including JNKs and p38 MAPK, are regulated by members of the Rho-family and are also sensitive to redox modulation [113]. JNKs and their downstream target c-Jun are stimulated by $H_2O_2$ during ROS -induced apoptosis of endothelial cells. The Akt kinase, a downstream effector of phosphoinositide 3-kinase (PI3-kinase), is involved in antiapoptotic signalling [114] and regulated by ROS in AngII-stimulated SMCs [115]. In endothelial cells, activation of Akt has been proposed to facilitate a protective effect against shear stress and apoptosis [108] and to reduce eNOS activity by posttranslational modification [71]. Interestingly, Rac1 is a further downstream target of PI3-kinase [108] and, thus, a stimulation of PI3-kinase might initiate Rac1-mediated NAD(P)H oxidase activation resulting in a simultaneous formation of ROS and Akt stimulation.

A growing body of evidence also suggests that ROS affect various protein tyrosine kinases (PTKs). Fyn, a member of the src-family, has been shown to be responsible for $H_2O_2$-mediated activation of Ras and 90kDa of ribosomal S6 kinases (p90RSK), in addition, Fyn might regulate Ras and, in turn, p90RSK, in a redox-sensitive manner [116]. Hence, $H_2O_2$-mediated BMK1 activation requires c-Src (p60Src) [13, 116, 117] indicating that the c-Src / BMK1 signalling pathway is redox-sensitive. In addition to p60Src, p56Lck, p59Fyn, Syk, ZAP-70 [13] and the $Ca^{2+}$-sensitive proline-rich tyrosine kinase Pyk2 [118] are also affected by ROS. In ROS-treated endothelial cells tyrosine phosphorylation of the Pyk2 downstream

**Table 1.** Redox-sensitive Protein kinases / phosphatases. Effects due to $H_2O_2$; taken from Allen and Tresini [222]

| Kinase/Phosphatase | Cell type | Effect |
|---|---|---|
| ERK pathway (MAP-kinase) | Bovine tracheal monocytes | Phosphorylation of ERK-1/2 |
| | Human neutrophils | Increased phosphorylation of ERK-1/2 |
| | Jurkat T cells | Activated ERK-1/2 |
| | NIH3T3 | Activated ERK-2 |
| | Rat alveolar macrophages | ERK-1/2 phosphorylation |
| | Rat arterial smooth muscle cells | ERK-1/2 phosphorylation |
| | Rat cardiac myocytes | Activated ERK-2 |
| | Rat smooth muscle (A7r5) | ERK-1/2 phosphorylation |
| | Rat vascular smooth muscle | ERK-1/2 phosphorylation; No effect |
| | Rat ventricular myocytes | Activated ERK-1/2 |
| BMK-1/ERK-5 | Mouse fibroblasts | Activated |
| | Rat vascular smooth muscle | Activated |
| | Human vascular smooth muscle | Activated |
| | Human umbilical vein endothelial cells | Activated |
| | Fibroblasts | Activated |
| JNK1/SAPK (MAP kinase) | Chicken beta cells | Activated |
| | Human fibroblasts | Activated |
| | Human vascular smooth muscle | Increased activity |
| | NIH3T3 | Increased phosphorylation |
| | Rat arterial smooth muscle cells | Increased phosphorylation |
| | Rat ventricular myocytes | Activated |
| p38 (MAP kinase) | Human umbilical vein endothelial cells | Activated |
| | Mouse NIH 3T3 | Increased phosphorylation |
| | Rat alveolar macrophages | Increased phosphorylation |
| | Rat arterial smooth muscle cells | Increased phosphorylation |
| | Rat vascular smooth muscle | Increased phosphorylation |
| MEK | Bovine tracheal monocytes | Increased activity of MEK1 |
| Raf-1 | Bovine tracheal myocytes | Increased activity |
| PKC | Human Jurkat T cells | Increased activity |
| $p56^{lck}$ | Human Jurkat T cells | Phosphorylation Tyr-394, Tyr505 |
| | Rat 208F fibroblasts | Stimulated activity |
| | Increased phosphotyrosine | |
| $pp60^{src}$ | Pig vascular endothelium | Increased phosphotyrosine |
| Lyn | Chicken beta cells (DT 40) | Stimulated activity |
| SYK | Chicken beta cells | Activated |
| ZAP-70 | T lymphocytes | Activated |
| | Jurkat T cells | Activated |
| Phosphatase Ser/Thr | Human erythrocytes | Increased activity of membrane bound form |
| | Sheep erythrocytes | Increased activity of membrane bound form |
| Phosphatase Tyr | Human fibroblasts (EK4) | Increased mRNA |

target p130cas, of a focal adhesion kinase (p125FAK) and of paxillin is increased [119]. The cytosolic tyrosine kinase p125FAK, a non-receptor protein-tyrosine kinase, plays a central role in the regulation of the actin cytoskeletal organisation by phosphorylating components of focal adhesion, such as tensin, paxillin and talin. Furthermore, p125FAK regulates interactions of integrins with the cytoskeleton and with the extracellular matrix or both. Thus, stimulation of p125FAK by ROS mediates reversible changes in cell shape and morphology, reorganisation of the cytoskeleton and redistribution of cell-surface adhesion proteins [119]. The involvement of high glucose concentration in ROS and tyrosine kinase activation hyperglycaemia is further supported by findings that genistein, an antioxidant and non-selective inhibitor of tyrosine kinases, prevented glucose-mediated atherogenic modification of low density lipoprotein [120].

Besides a direct activation of PTKs by ROS, protein tyrosine phosphatases (PTPs) seem to be prime candidates for ROS signalling. Most PTPs contain conserved cysteine residues within their active domains [108]. Their oxidation might affect the biological activity of the phosphatase. Attenuated PTP activity shifts the balance of protein tyrosine phosphorylation and dephosphorylation towards a status of enhanced protein tyrosine phosphorylation of cellular targets of PTKs, such as ERKs and SAPKs.

From the above it is apparent that a variety of ROS-sensitive protein kinases, such as PKC and PTKs, is involved in vascular cell dysfunction. Even isoforms of these enzymes could constitute promising

selective targets for therapeutic intervention against diabetes-associated vessel complications.

*Matrix metalloproteinases (MMPs).* MMPs, also termed matrixins, are members of a family of $Zn^{2+}$- and $Ca^{2+}$-dependent endopeptidases, which are essential for cellular migration and tissue remodelling [121]. They have been shown to play an important role in atherosclerosis and angiogenesis, whereas little is known about the effects of hyperglycaemia on MMP regulation in vascular cells [4]. The expression and activity of 92-kDa (MMP-9) gelatinase are increased in vascular tissue and plasma of diabetic models [4] and in cultured bovine aortic ECs (but not in SMCs or macrophages) after long-term exposure to high D-glucose [4]. The rise in enzyme activity is markedly reduced by treatment with antioxidants such as polyethylene glycol-SOD, superoxide dismutase and N-acetyl-L-cysteine, but not by inhibitors of PKC [4], although PMA, an PKC activator, increases MMP-9 expression in various cell types, including vascular endothelial cells [122]. The MMP-9 promoter region contains binding sites for the redox-sensitive transcription factors NF-$\varkappa$B and AP-1 [123] indicating their involvement in ROS-induced MMP-9 transcription and activity [124].

MMP-2 and MMP-9, which are regulated by oxidative stress [125], are actively synthesised in atheromatous plaques [126] and appear to contribute to monocyte invasion and vascular SMC migration. Therefore, any derangement of MMP regulation is considered a critical factor in the development of vascular complications [127] including acute myocardial infarction and unstable angina [128]. It has also been established that MMP-9 activity is required for angiogenesis and neovascularisation, which constitute important elements in the mechanisms of plaque progression in atherosclerosis [129].

So far no causal link has been provided between the disruption of the cytoskeleton and the expression of MMPs. A recent study showed disruption of the actin cytoskeleton induced with an antibody against $\alpha_5\beta_1$ integrin increased expression of MMP-1 in rabbit synovial fibroblasts [130]. Possibly Rac becomes activated during cytoskeletal reorganisation resulting in increased NAD(P)H oxidase activity and recruitment of NF-$\varkappa$B [131]. Thereby IL-1$\alpha$, an autocrine inducer of MMP-1 expression, is induced. Alternatively, in endothelial cells the disruption of the cytoskeletal network has been shown to affect subcellular $Ca^{2+}$ signalling [132, 133] subsequently regulating a variety of signal transduction pathways and transcription factors [134, 135, 136, 137] (Fig. 3). Thus, a contribution of altered spatial $Ca^{2+}$ signalling in MMP expression in response to cytoskeleton disruption appears possible.

Any such enhanced MMP activity could be critical for diabetic microvascular complications. Although

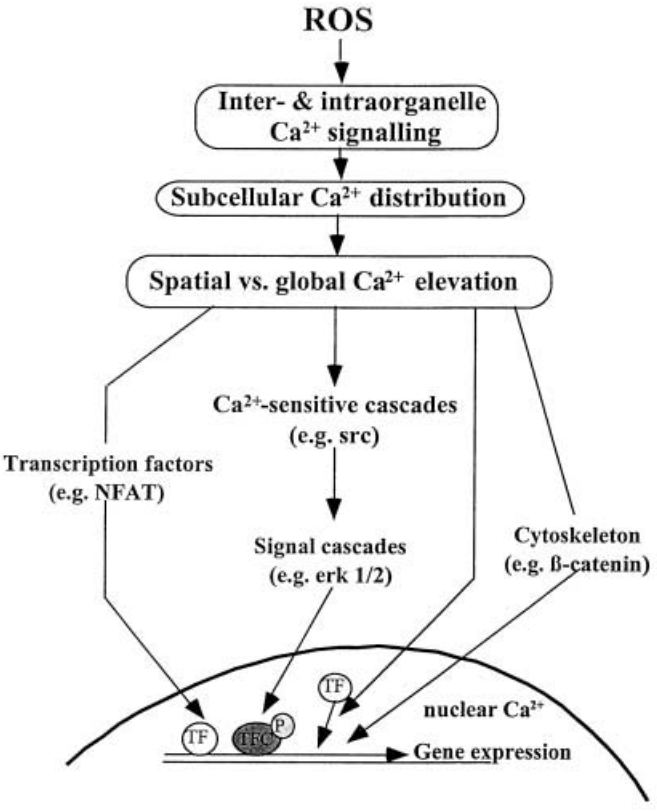


**Fig. 3.** Possible effects of ROS on intracellular $Ca^{2+}$ handling and subcellular $Ca^{2+}$ distribution and its consequences for $Ca^{2+}$ modulated gene expression. The production of ROS due to diabetic-associated risk factors initiates changes in inter- and intra-organelle $Ca^{2+}$ handling and disturbances in the subcellular $Ca^{2+}$ distribution. Due to the lack of the formation of locally insulated $Ca^{2+}$ gradients for specific activation of $Ca^{2+}$-sensitive gene regulation, the whole $Ca^{2+}$-sensitive transcription machinery is ubiquitous activated, resulting in uncontrolled activation of $Ca^{2+}$-sensitive gene expression

the exact mechanisms that link diabetes and ROS with MMP expression are not fully understood, additional studies are necessary to further define MMPs as potential therapeutic targets in diabetic blood vessel dysfunction.

*Ion homeostasis.* Ion channels play a crucial role in vascular homeostasis. $K^+$-current that hyperpolarises the cell reduces $Ca^{2+}$ L-type channel activity and relaxes smooth muscle cells (SMCs), whereas the inhibition of $K^+$ channels facilitates the activation of the $Ca^{2+}$ L-type channels and therefore SMCs contraction [138]. In endothelial cells (ECs) the impact of $K^+$ channels on $Ca^{2+}$-entry is opposite to that found in SMCs, because $K^+$ channel-mediated membrane hyperpolarisation facilitates $Ca^{2+}$ entry through so called store-operated $Ca^{2+}$ channels (i.e. non-voltage gated $Ca^{2+}$ channels). However, an increase in endothelial $Ca^{2+}$ concentration primarily activates eNOS, prostacyclin synthesis and formation of endothelium-derived hyperpolarising factor(s) (EDHF(s)),

leading to blood vessel relaxation [139]. Recently, endothelial $K^+$ outward currents were shown to constitute a EDHF-like mechanism due to locally raised $K^+$ concentrations in the direct vicinity of smooth muscle $K^+$ channels [140]. Thus, while the effect of $K^+$ channel activation to cellular $Ca^{2+}$ concentration differs in ECs and SMCs, the impact of $K^+$ channels for global vascular response (i. e. relaxation, contraction) is identical. The potentially diminishing effect of diabetes mellitus on $K^+$ channel activity or activation involved in EDHF-mediated blood vessel relaxation has been shown in streptozotocin-induced diabetic rats [141, 142]. Furthermore, reduced activation of ATP-sensitive $K^+$ channels leading to membrane depolarisation and vasoconstriction has also been described in diabetic rats [143, 144] indicating that besides $NO^\bullet$ the formation and the effect of EDHF can also be impaired in diabetes.

In ECs the effect of ROS on ion channels (Fig. 2) and subsequent ion signalling considerably relies on the oxygen species and can be varied depending on acute as opposed to chronic actions. Peroxides such as $H_2O_2$ or tertiary-butylhydroperoxide (t-BuOOH) appear to reduce the $Ca^{2+}$ influx [145]. Possibly linked to the duration of treatment and usage of media supplements, hyperglycaemia was found to enhance [146] or to impair [147] intracellular $Ca^{2+}$ concentrations upon agonist stimulation due to the accumulation of $O_2^{\bullet-}$. In line with the latter findings, endothelial $Ca^{2+}$ signalling was found to be impaired in diabetic mice [148, 149]. Remarkably, in myocytes hyperglycaemia was found to increase cytosolic free $Ca^{2+}$ [150], while chronic diabetes impaired L-type $Ca^{2+}$ channel activity [151].

Hence, in ECs t-BuOOH has been shown to increase the opening probability of non-selective cation channels of 30 pS [152] and of Trp3 [153], resulting in membrane depolarisation. In ventricular myocytes, extracellularly produced ROS activate $Ca^{2+}$-dependent nonselective cation channels without any change in cytosolic $Ca^{2+}$ concentration [154]. Furthermore, membrane depolarisation can be due to an inhibition by $H_2O_2$ of the intermediate conductance $K_{Ca}$ channel [155]. In human umbilical vein endothelial cells an enhanced $O_2^{\bullet-}$ production by Rac-dependent NAD(P)H oxidase activation occurs as a result of membrane depolarisation [156]. As pointed out above, the formation of $O_2^{\bullet-}$ is of particular impact in endothelial cells regarding the local $NO^\bullet$ formation because these radicals can react to form $ONOO^-$ which, in turn, inhibits $Ca^{2+}$ influx [157] and the activity of $BK_{Ca}$ channels [158]. Thus, it is reasonable that during certain forms of oxidative stress (peroxides, $ONOO^-$) membranes become depolarised resulting in an attenuated driving force for $Ca^{2+}$ and in reduced $Ca^{2+}$-entry. In view of the importance of $Ca^{2+}$-entry for eNOS activation [159, 160], one could assume that t-BuOOH-mediated membrane depolarisation reduces eNOS activation in response to $Ca^{2+}$ elevating agonists.

There is increasing evidence that, in addition to channels in the plasma membrane, $Ca^{2+}$ channels in organelles (i. e. endoplasmic reticulum and mitochondria) are common targets of several forms of ROS. The mechanisms by which ROS affect $Ca^{2+}$ unloading of the endoplasmic reticulum are not clear but could be related to changes in the activity of sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATP-ases (SERCAs) [161], $IP_3$ receptors [162] and ryanodine receptors (RyR) [163]. The latter is stimulated in skeletal muscle by $H_2O_2$ [164] and in cardiac muscle by $HO^\bullet$ [165]. Furthermore, oxidants (e. g. t-BuOOH) seem to reversibly increase the permeability of the inner mitochondrial membrane by pore formation, leading to $Ca^{2+}$ release from the mitochondria [166, 167]. Most of these studies have relied essentially upon the use of isolated mitochondria loaded with $Ca^{2+}$, and then exposed to oxidants. On the contrary, the work of Jornot et al. [168] in intact cells demonstrated higher $Ca^{2+}$ concentration in the mitochondria, which was preceded by a rise in cytosolic free $Ca^{2+}$ concentration. This ROS ($H_2O_2$, or $O_2^{\bullet-}$) initiated increase in mitochondrial $Ca^{2+}$ concentration resulted from a transfer of $Ca^{2+}$ by the electrogenic uniporter, and a decrease in the rate of $Ca^{2+}$ efflux from the mitochondria via the $Ca^{2+}/Na^+$ exchanger.

In view of the importance of ion channels for vascular cell function, their contribution to diabetes-associated complications needs more attention in order to understand whether ion channels are attacked by ROS directly or by one of their downstream targets. Increased ROS production and subsequent alterations in intracellular $Ca^{2+}$ concentration in endothelial cells, smooth muscle cells and platelets [169] are the cause of many cardiovascular diseases. While most studies are focussed on global $Ca^{2+}$ signalling [170, 171, 172, 173], we could describe changes in subcellular (perinuclear and subplasmalemmal) $Ca^{2+}$ distribution in smooth muscle cells from diabetic patients [174] (Fig. 3). This study is in line with findings in the rat tail artery, where hyperglycaemia was found to initiate alterations of cytosolic $Ca^{2+}$ signalling [175]. This was accompanied by ROS-triggered restructuring of the cytoskeleton and the endoplasmic reticulum. Thus, under pathological conditions accompanied by increased ROS production, changes in subcellular $Ca^{2+}$ signalling could represent an early target for altered endothelial function that results in changes in cell function (e. g. eNOS, [51, 53, 70, 176]) and gene expression [37, 177].

*Transcriptional regulation.* In diabetic patients activation by hyperglycaemia, AGEs, oxidised LDL and ROS of the inducible transcription activator NF-$\varkappa$B could promote atherosclerosis and its rapid progression [28, 84, 178, 179]. NF-$\varkappa$B that has been shown to

**Table 2.** Redox-sensitive Regulatory factors. Effects due to $H_2O_2$; taken from Allen and Tresini [222]

| Gene or protein | Cell type | Effect |
|---|---|---|
| NF-κB | Human endothelial cells (ECV304) | Activated |
| | Human Jurkat T-cells | Activated |
| | Human microvascular endothelial cells | Increased DNA binding |
| | Human T cells (peripheral) | Decreased DNA binding |
| | Jurkat T cells | Activated |
| | Mouse fibroblasts (3T3) | Activated |
| | Mouse macrophages (J774A.1) | Activated |
| | Pig vascular endothelium | Increased DNA binding |
| | Rat heart endothelial cells | Activated |
| IκB | Human endothelial cells (ECV304) | Stimulated degradation of IκBα |
| NF-AT (protein) | Jurkat T cells | Decreased transcriptional activation by NF-AT |
| | Human T cells (peripheral) | Decreased DNA binding |
| AP-1 | Human microvascular endothelial cells | Increased DNA binding |
| | Human T cell (peripheral) | Decreased DNA binding |
| | Big vascular endothelium | Increased DNA binding |
| | Rat embryo glial cells | Increased binding to TRE |
| | Rat heart endothelial cells | Activated |
| c-jun | Human fibroblasts (DET-551) | No effect |
| | Human fibroblasts (IMR-90) | No effect |
| | Human fibroblasts (WI-38) | No effect |
| | Jurkat T cells | Increased mRNA |
| | Rat vascular smooth muscle | Increased mRNA |
| Jun protein | Rat vascular smooth muscle | Increased protein |
| c-fos | Rat vascular smooth muscle | Increased mRNA |
| Fos protein | Rat vascular smooth muscle | Increased protein |
| CREB | Rat embryo glial cells | Increased binding to CRE |
| c-myc | Rat vascular smooth muscle | Increased mRNA |

be activated by ROS by Rac1-transmitted NAD(P)H oxidase activation, is linked to endothelial dysfunction and vascular inflammation [108] as well as proliferation in vascular smooth muscle cells, a mechanism critically involved in neo-intima formation. From this it appears that NF-κB is crucially involved in the pathogenesis of atherosclerotic lesions by switching on specific target genes, such as vascular cell adhesion molecules, intercellular adhesion molecules and E-selectin [179]. In bovine endothelial cells hyperglycaemia yielded such activation of NF-κB via a PKC-dependent mechanism [180]. In the same cell type, AGE products that can initiate oxidative stress [181, 182] activated NF-κB by a mechanism sensitive to alpha-lipoic acid [183]. Increased NF-κB activity seems to be essential for the augmented leucocyte-endothelial interaction during hyperglycaemia [184]. Glycated serum albumin and hyperglycaemia were also found to stimulate NF-κB in smooth muscle cells [185, 186] and mesangial cells (high D-glucose) [187] by the generation of ROS. Furthermore, the involvement of poly(ADP-ribose) polymerase has been reported in the activation of NF-κB by hyperglycaemia [188]. These studies are further supported by the report that insufficient glycaemic control increases NF-κB binding activity in peripheral blood mononuclear cells isolated from patients with Type I (insulin-dependent) diabetes mellitus [189].

Notably, incubation of HUVECs with high D-glucose increases rapidly the generation of ROS accompanied by NF-κB activation, which can be prevented by antioxidants (tocopherol, SOD-mimetic) (Table 3). The formation of ROS was additionally inhibited by the specific eNOS inhibitor L-nitroarginine (L-NNA) [10] and DPI [33], whereas inhibitors of cyclooxygenase and lipoxygenase had no influence [28]. This indicates that high D-glucose leads to an increase in generation of ROS (mainly $O_2^{\bullet -}$ and $NO^{\bullet}$ [28]) and activates NF-κB by a D-glucose specific and eNOS- and PKC-dependent mechanism. Hence, Du et al. [178] suggested $ONOO^-$ as the mediator of the effects caused by high D-glucose on endothelial cells. Furthermore, $ONOO^-$ has been shown to activate NF-κB [178].

ROS-mediated changes in the architectural organisation of the cytoskeleton and $Ca^{2+}$ containing organelles yields marked alterations in cellular $Ca^{2+}$ homeostasis (see above). This redistribution might largely contribute to the changes in cell function under pathological conditions that accompany increased ROS production. Moreover, changes in subcellular $Ca^{2+}$ distribution (Fig. 3) could reorganise the architecture of the cytoskeleton, modulate $Ca^{2+}$-sensitive/-activated transcription factors and signal cascade enzymes resulting in altered gene expression [136, 190, 191, 192] (for review see [135]). In line with these data in beta cells, in endothelial cells the

**Table 3.** Redox-sensitive Regulatory factors. Effects due to Antioxidants; taken from Allen and Tresini [222]

| Antioxidant | Gene or protein | Cell type | Effect |
|---|---|---|---|
| NAC | NF-$\varkappa$B | Human endothelial cells (ECV304) | Blocked activation by $H_2O_2$ |
| | I$\varkappa$B | Human Jurkat T cells | Prevented $H_2O_2$ activation |
| | AP-1 | Jurkat T cells | Decreased activation by TNF-$\alpha$ |
| | Jun protein | Mouse fibroblasts (3T3) | Blocked activation by TNF |
| | c-fos | Jurkat T cells | Blocked phosphorylation and degradation of I$\varkappa$B$\alpha$ after TNF-$\alpha$ stimulation |
| | Fos protein | Rat vascular smooth muscles<br>Rat vascular smooth muscle<br>Human fibroblasts (WI-38)<br>Rat vascular smooth muscle | Blocked activation by serum<br>Blocked increase stimulated by serum<br>Increased transcription<br>Blocked increase stimulated by serum |
| Tocopherol | NF-$\varkappa$B | Human T lymphocytes (J. Jhan)<br>Human monocytes (937) | Blocked PMA and TNF activation<br>Blocked PMA and TNF activation |
| NGA | NF-$\varkappa$B | Human T lymphocytes (J. Jhan) | Blocked PMA and TNF activation |
| | Jun protein | Human monocytes (937) | Blocked PMA and TNF activation |
| | c-fos | Rat vascular smooth muscle | Blocked $H_2O_2$, AA effects |
| | Fos protein | Human fibroblasts (WI-31)<br>Rat vascular smooth muscle<br>Rat vascular smooth muscle | Increased transcription<br>Blocked $H_2O_2$, AA<br>Blocked $H_2O_2$, AA |
| Phenolic antioxidant BHA | NF-$\varkappa$B | Human endothelial cells (ECV304)<br>Human T lymphocytes (J. Jhan)<br>Human monocytes (937) | Blocked TNF-$\alpha$ effects<br>Blocked PMA and TNF activation<br>Blocked PMA and TNF activation |

same effects could be observed under diabetic conditions [28, 54, 193, 194, 195, 196, 197]. Thereby, the impact of $Ca^{2+}$ on gene expression exceeded its role in the nuclear envelope, where $Ca^{2+}$ is essential for modulating immediate early genes by modulating the DNA-binding of transcription factors (e.g. cAMP-responsive element binding protein CREB [191, 192, 198, 199, 200]).

$Ca^{2+}$ triggers the activation of transcription factors such as the nuclear factor of activated T cells (NFAT) [201, 202, 203, 204, 205, 206] and the serum response factor-related proteins (RSRF or the myocyte enhancer factor 2 (MEF2) [207]) by calcineurin [208] or $Ca^{2+}$-sensitive kinases (e.g. Cam-kinase IV [207]). In the case of NFAT, the $Ca^{2+}$/calmodulin-activated protein phosphatase-2B (calcineurin) dephosphorylates NFAT resulting in its translocation into the nucleus [208] to form a heteromeric transcriptional activator complex with activator-protein-1 (AP-1) and initiates gene expression [202]. In vascular smooth muscle cells NFAT activation by increasing cytosolic free $Ca^{2+}$ concentration depends on the patterns of the $Ca^{2+}$ signalling observed in response to the compound tested. Angiotensin II and thrombin, which rapidly but transiently increase $Ca^{2+}$ concentration, activate NFAT-mediated transcription rather weakly [206]. In contrast, the platelet-derived growth factor BB (PDGF-BB) yields a higher activity in NFAT-mediated transcription despite the smaller, slower but longer lasting $Ca^{2+}$ elevation. Thus, depending on the kinetics of bulk $Ca^{2+}$ increases and that of localised $Ca^{2+}$ increases, $Ca^{2+}$ modulates NFAT-mediated gene expression. In endothelial cells, regulation by oscillation frequency of agonist-stimulated NF-$\varkappa$B transcriptional activity has been demonstrated [137]. Remarkably, NF-kB transcriptional activity was attenuated by a reduction of the frequency of $Ca^{2+}$ oscillation at constant amplitude of $Ca^{2+}$ increases.

Hence, cytosolic $Ca^{2+}$ stimulates mitogenic signal cascades that induce gene expression (e.g. erk-1/2 [199]; src [116]) and the rearrangement of cytoskeletal proteins ([209], e.g. beta catenin [210, 211], or zyxin [212]). Cytosolic signal cascades might be modulated by the patterns of the cytosolic $Ca^{2+}$ signalling (e.g. transient or long lasting $Ca^{2+}$ increases compared to spiking) that, in turn, is controlled by spatial $Ca^{2+}$ distribution (e.g. subplasmalemmal $Ca^{2+}$ [213, 214, 215, 216, 217]).

Many ROS-sensitive protein kinases (Table 1 Fig. 2) mentioned above further activate or modulate transcription factors as important downstream targets (Table 2; Table 3).

P90RSK that is regulated by its upstream regulator ERK1/2 [218] as well as via Fyn and Ras [116], phosphorylates c-Fos [13], Nur77 [219] and Ik-B, the latter resulting in NF-kB activation [13, 220]. Recently, p90RSK and the closely-related rsk genes have been shown to affect the activity of CREBs [221], while the regulation of AP-1 by ROS involves ERK1/2 and JNK in a cell and stimulus specific manner [13].

## Conclusion

The generation of ROS in diabetes mellitus represents a crucial phenomenon that links high D-glucose, AGEs and glycated (lipo-) proteins with particular changes in cell structure and function. In this review we presented some targets of ROS that are rarely discussed and considered in the evaluation of vascular dysfunction in diabetes. That does in no way weaken the importance of other cellular targets of ROS (e.g. PKC, lipids), but might add some new perspectives and targets worthy to be assessed in more detail in diabetes-associated vascular complications. Although there is strong evidence for an activation of NF-kB and AP-1, additional studies will be indispensable to assess the whole range of ROS-activated transcription factors.

*Sources.* This review is based on the selected literature published in the English language during the last decade. The authors own sources were integrated with sources that resulted from PubMed searches using a search profile containing the words "radicals, peroxide, superoxide anions, oxidative stress" either alone or in conjunction with "diabetes, hyperglycaemia".

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