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Regional Differences in the Expression of Nitric Oxide Synthase and Specific Receptors in the Vascular Tissues of Control and Diabetic Rabbits: A Pilot Study

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Abstract. *Background: Atherosclerosis can influence the expression of endothelial nitric oxide synthase (eNOS) as well as endothelin-1 (ET-1) and 5-hydroxytryptamine (5HT; serotonin) receptors. Diabetes has an effect on the onset, severity and pattern of atherosclerosis with a predilection for more distal arteries. We aimed to identify regional differences in the distribution of eNOS activity, ET-1 and 5HT receptors in vascular tissues obtained from control and diabetic rabbits. Materials and Methods: The mid abdominal aorta, right renal and right femoral arteries were harvested from 12 adult rabbits (6 months old, 3-3.9 kg); 8 controls and 4 diabetic (induced using alloxan 7 months previously). Samples were stored in liquid nitrogen for Western immunoblotting for eNOS as well as ET-1 and 5HT receptors. Results: Significant differences were found in the distribution of eNOS, ET-1 and 5HT between the aorta, renal and femoral arteries in the controls. The number of ET-1 receptors was significantly higher (aorta; p=0.016, renal; p=0.004, femoral; p=0.05,) whereas, the expression of eNOS was significantly lower (aorta; p=0.004, renal; p=0.004, femoral; p=0.008) when comparing arteries from normal rabbits with these from diabetics ones. The number of 5HT receptors was higher in arteries from diabetic rabbits but this was not statistically significantly. Conclusion: The "regional" distribution of eNOS activity as well as ET-1 and 5HT receptors in control rabbits varies significantly according to the vessel assessed. Further studies are needed to evaluate the effect of blocking these receptors (e.g. on the risk of re-stenosis). Regional receptor differences may explain why diabetes is linked with a predilection for atherosclerosis (and possibly calcification) in distal arteries.*

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The endothelium plays an important role in the regulation of blood flow and in maintaining vascular tone by releasing factors such as endothelin-1 (ET-1) and nitric oxide (NO). Increased vasomotor tone is partly due to the reduced bioavailability of the endogenous vasodilator, endothelium-derived NO (1, 2). Furthermore, the vessel wall also produces vasoconstrictors that may contribute to the heightened coronary vasomotor tone in patients with atherosclerosis.

The risk for all forms of cardiovascular disease (CVD) is substantially increased with type 1 and type 2 diabetes (3). Furthermore, endothelial dysfunction and increased arterial stiffness predate symptomatic CVD in diabetes and the metabolic syndrome (4). Both these dysfunctional properties of the arterial circulation have a common determinant in abnormal metabolism of vasoactive factors, such as 5 hydroxytryptamine (5HT; serotonin) but in particular of NO.

NO is catalysed from the amino acid L-arginine and molecular oxygen by endothelial NO synthase (eNOS). eNOS is primarily expressed in endothelial cells and is an important regulator of vascular function. It also exerts an anti-inflammatory influence, inhibits platelets adhesion and aggregation, and prevents smooth muscle cell proliferation and migration. During atherogenesis, NO plays a protective role by reducing the proliferation and migration of smooth muscle cells (5), by inhibiting the adhesion of platelets (6-8) and leukocytes (9) to the endothelium, and by scavenging superoxide radicals (10). Several lines of evidence link endothelial dysfunction, characterized by decreased bioavailability of NO, with the development of pathological conditions such as heart failure, hypertension, diabetes and atherosclerosis (5, 11-15).

ET-1 (a 21-amino acid peptide) is one of the most potent vasoconstrictors produced in the arterial wall. Although only scant immunoreactive ET-1 is present in the endothelium of

normal human coronary arteries, more ET-1 is present throughout the thickened intima of atherosclerotic human coronary arteries (16, 17). Whether this immunoreactive ET-1 contributes to the exaggerated constriction of atherosclerotic human coronary arteries has not been established. The availability of ET-1 receptor antagonists has allowed the investigation of the role of ET-1 in the vasomotor control of the human circulation *in vivo* (18-21). To date, two types of ET-1 receptor have been identified: ETA and ETB (22-25). In vascular smooth muscle, ETA is the most abundant receptor with activation causing contraction irrespective of species (22, 23).

5HT is synthesized in the enterochromaffin cells of the gastrointestinal tract and is released into the blood stream and stored in platelet dense granules (26). Platelets participate in the development of atherothrombosis; in fact, at a site of endothelial lesion, platelets aggregate and secrete 5HT which may be involved in several vascular actions (*e.g.* thrombosis and atherogenesis) (27, 28). Furthermore, 5HT can induce: vasoconstriction in the presence of endothelial damage (29). These actions of 5HT are mediated *via* specific 5HT receptors, which are currently classified into 7 5HT subtypes. Vasoconstriction to 5HT is predominantly mediated *via* smooth muscle 5HT2 receptors and/or 5HT1 receptors (30, 31).

In this pilot study we aimed to identify regional differences in the distribution of eNOS activity as well as ET-1 and 5HT receptors in different vascular tissues obtained from control and diabetic rabbits.

Materials and Methods

The study was conducted under a project license granted by the Home Office in accordance with the Animals (Scientific Procedures) Act 1986. All animals were kept in a temperature controlled environment with a 12 h light-dark cycle.

Male Adult New Zealand white rabbits [(n=12) Highgate Farm, Market Rasen, Lincolnshire, UK] were used in this study, 4 rabbits received an intravenous injection *via* the lateral ear vein of alloxan (Sigma) at a start dose of 65 mg/kg in a volume of 1 ml saline per kg; to induce diabetes. Control animals (n=8) were injected with saline vehicle alone. Diabetic animals received 3 subcutaneous injections of 10 ml of 50% glucose, 4 h apart on the first day following the alloxan injection. A final glucose injection (10 ml of a 50% glucose solution) was administered on the morning (7:30 am) of the second day. This procedure counteracts the hypoglycemia caused by insulin release from necrosed pancreatic beta cells due to the acute action of alloxan. The diabetic rabbits were fed *ad libitum* with SDS standard rabbit chow (SDS, Whitham, UK) and allowed free access to water. Blood samples were collected a week following the alloxan injection to confirm diabetes.

Surgical procedure. After 7 months of alloxan treatment, rabbits were taken to theatre. Under sterile conditions, a median laparotomy was performed. After transposition of the viscera towards one side, careful dissection was used to expose the abdominal aorta. The aortic segment just below the diaphragm was also exposed. A segment of the mid aorta was taken together with the right renal artery. Mass closure using silk was performed to close the abdomen. Another longitudinal incision to the right groin was performed to expose the femoral artery. Samples were collected and stored in liquid nitrogen for Western immunoblotting.

Western immunoblotting protocol. Samples were homogenised as previously described (32). Samples of the arteries were incubated in bovine serum albumin (BSA) with diluted goat antibody [from Vector Laboratories Ltd (Burlingame, CA, USA)]. Then centrifuged at 8,000 xg for 10 min at 4˚C, and the supernatants were used as tissue extracts.

A 2 μl aliquot of the supernatant was used for protein determination using a commercial kit (Bio-Rad *DC* Protein Assay, Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire UK).

Samples were then diluted in Laemmli buffer to a concentration of 80 μg and heated at 90˚C for 10 min using a dry heating block. Protein (80 μg) for each sample was loaded onto a 12 cm 7.5 % w/v SDS-polyacrylamide resolving gel with a 4% w/v SDS-polyacrylamide stacking gel. Dilute the Running Buffer (10X) 1:10 in dH_2O (*i.e.* add 100 ml of concentrate to 900 ml dd H_2O). We then diluted transfer buffer to 1X and made up to + 20% methanol (*i.e.* add 200 ml of methanol to 800 ml of 1X transfer buffer). Electrophoresis of the samples was performed at 150 V, until the bromophenol blue marker band had migrated the length of the gel, using a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK).

The gel was then washed in transfer buffer (running buffer containing 20% v/v methanol) and proteins were electrophoretically transferred onto a nitrocellulose membrane at 30 V overnight at 4˚C or room temperature for 1.5 h.

Non-specific antibody binding was blocked with 5% w/v non-fat dried milk containing 0.05% v/v Tween 20 for 2 h at room temperature on an orbital shaker.

The membrane was washed in TBS-T for 10 min x 3. Before being probed with rabbit anti-NOS polyclonal antibody (Transduction) (1/1000 dilution in TBS) for 2 h at room temperature. This step was repeated. The membrane was then incubated with secondary antibody goat anti-rabbit biotinylated (Vector) (1/1000).

The membrane was washed again 3 times in TBS-T before incubation in substrate solution (0.1 M Tris-HCl, pH 8.2 containing 20 ml/ml of 20 mM naphthol AS-BI phosphate in dimethylformamide and 1 mg/ml Fast Red RC salt).

The membrane was finally washed in distilled water before being scanned using a densitometer (UN-SCAN-IT gel, Silk Scientific, Inc. P.O. Box 533 Orem, Utah 84059 USA). Units used to express results are μg per μl of solubilised protein.

Statistical analysis. The paired nonparametric Wilcoxon test was used to compare the differences in the distribution of receptors in control vascular tissues. The unpaired nonparametric Mann-Whitney test was also used to compare the differences in the distribution of receptors in control and diabetic vascular tissues.

All tests were performed using the software program GraphPad Prism (version 4.03, Sad Diego, California USA); significance was defined as $p<0.05$.

Table I. *Distribution of endothelial nitric oxide synthase (eNOS) (Controls vs. Diabetics) expressed as μg per ml of protein (see text).*

Rabbits	Aorta		Renal		Femoral	
	Con	DM	Con	DM	Con	DM
1	75	36	20	16	164	49
2	73	17	87	12	186	19
3	73	17	86	10	187	10
4	59	30	72	42	199	70
5	44		54		157	
6	38		85		280	
7	36		89		142	
8	55		77		185	
Mean	57	25	71	20	187	37

DM *vs*. Con	*P*-value
Aorta	0.004
Renal	0.004
Femoral	0.008

Table II. *Distribution of endothelin (ET-1) receptors (Controls vs. Diabetics) expressed as μg per ml of protein (see text).*

Rabbits	Aorta		Renal		Femoral	
	Con	DM	Con	DM	Con	DM
1	25	34	40	145	108	152
2	39	44	63	367	203	920
3	13	29	39	128	177	289
4	12	52	115	490	263	565
5	28		57		134	
6	12		59		112	
7	15		43		143	
8	11		63		281	
Mean	19	40	60	282	178	481

DM *vs.* Con	*P*-value
Aorta	0.016
Renal	0.004
Femoral	0.051

Results

Distribution of receptors and eNOS activity in control rabbits. Significant differences were found in eNOS activity between the aorta *vs.* femoral ($p=0.007$) and renal *vs.* femoral ($p=0.008$) arteries. However, the difference between the aorta and renal arteries was not significant ($p=0.19$).

Significant differences were found in the distribution of ET-1 receptors between the aorta *vs.* renal ($p=0.007$) and aorta *vs.* femoral ($p=0.008$) and renal *vs.* femoral ($p=0.007$) arteries in the control rabbits.

Significant differences were found in the expression of 5HT receptors between the aorta *vs.* femoral ($p=0.008$) and renal *vs.* femoral ($p=0.007$) arteries. However, the difference between the aorta and renal arteries was not significant ($p=0.22$).

The actual values are shown in Tables I-III.

Distribution of receptors and eNOS activity in diabetic rabbits and comparison with controls (Tables I-III). Any difference in eNOS activity as well as 5HT and ET-1 receptors in the arteries studied is not reported because of the small sample size.

Compared with controls, the expression of eNOS was significantly reduced (aorta: $p=0.004$, renal: $p=0.004$, femoral: $p=0.008$) in diabetic rabbits. On the other hand, the number of ET-1 receptors were significantly increased (aorta; $p=0.016$; renal; $p=0.004$; femoral; $p=0.05$) in diabetic rabbits. The number of 5HT receptors were higher (aorta $p=0.21$, renal: $p=0.07$, femoral: $p=0.57$) in diabetic rabbits but not significantly so.

Table III. *Distribution of serotonin (5HT) receptors (Controls vs. Diabetics) expressed as μg per ml of protein (see text).*

Rabbits	Aorta		Renal		Femoral	
	Con	DM	Con	DM	Con	DM
1	15	50	16	159	154	163
2	25	37	31	21	150	64
3	16	49	29	148	139	256
4	23	66	21	44	284	440
5	63		46		184	
6	68		23		102	
7	45		19		195	
8	36		17		145	
Mean	36	50	25	93	169	131

DM vs. Con	*P*-value
Aorta	0.211
Renal	0.072
Femoral	0.571

Discussion

eNOS in endothelial cells plays a crucial role in vascular tone and structure regulation (33). eNOS may also contribute to the vascular complications associated with diabetes (34). Diabetic mice that lack the eNOS gene demonstrate classic features of diabetic nephropathy with intrarenal vascular disease, and occasional microaneurysm formation. Evidence suggests that a relative deficiency in

eNOS may be one of the long-sought risk factors that are critical for the increased susceptibility for nephropathy in diabetic patients (34).

The potent vasoconstrictor-mitogen ET-1 has been implicated in the pathogenesis of vascular dysfunction in diabetes (35). A recent study showed that the effects of acute blockade of ET-1 receptors on the vascular function of superior mesenteric arteries and renal arteries reduces the vasoconstrictor responses in arteries of diabetic rabbits and these responses remained significantly greater in renal arteries (36). Another study showed that ET-1 levels were increased in the vasculature in untreated diabetic rats supporting the interpretation that the beneficial effects of bosentan (potent inhibitor of ETA/ETB receptors) were mediated, at least in part, by antagonism of ET-1 action on ET-1 receptors (35). ET-1 is causally linked to renal disorders characterized by increased renal vascular resistance. ET-1 receptor antagonists have been developed and tested in animal models with promising results (37). These data including our findings suggest that alterations in the production and/or action of ET-1 may play an important role in the pathogenesis of diabetic vascular disease.

Recent clinical studies suggest that 5HT-mediated platelet aggregation and vasoconstriction contribute to the onset of ischemic heart disease (38-40). Peripherally, numerous serotonergic receptors and receptor subtypes modulate a range of cardiovascular actions, including vasoconstriction, vasodilatation, platelet aggregation and positive inotropic and chronotropic effects (41). The potent and selective 5HT (1B/1D) receptor agonists, domitriptan and sumitriptan, have significant effects on vasoconstriction (42, 43). It has been suggested that the 5HT1B receptor, which is up-regulated by atherosclerosis, most likely mediated the augmented vasoconstriction effects of 5HT (44). Furthermore, a marked increase in 5HT into the coronary circulation was described in patients undergoing coronary angioplasty; the release of 5HT was followed by vasoconstriction distal to the dilated site (45, 46).

There is evidence that endothelium-dependent relaxation in the rat aorta is increased in the early stages of diabetes. Furthermore, exposure to a high concentration of glucose for 5 days was also shown to increase both the expression of eNOS and the production of NO in human aortic endothelial cells (47). Thus, plasma insulin and glucose levels in diabetes may regulate the production of NO and the expression of the mRNA for eNOS. However, no study of endothelial function in type 2 diabetes has directly assessed the expression of eNOS and the production of NO while focusing on time-dependent changes in endothelial function.

There is evidence that exogenous administration of ET-1 causes a dose-dependent augmentation of angioplasty-induced neointimal formation in the rat carotid artery (48). Recently, it was also shown that ET-1 receptor antagonists reduce neointimal formation in this animal model, suggesting that endogenous ET-1 is involved in angioplasty-induced lesion formation in the rat (49, 50). Using this model, the ET-1 selective antagonist, LU 135252, reduces percutaneous transluminal angioplasty-induced neointima formation.(51) Furthermore, ET-1 has been implicated in the pathogenesis of restenosis following percutaneous transluminal angioplasty. Moreover, a raised level of circulating immunoreactive ET-1 has been demonstrated in patients following this procedure (52) and there is more evidence of a time-dependent increase in ET-1 following balloon angioplasty in the rat (53). This tissue derived ET-1 may act on neighbouring receptors, situated on adjacent vascular smooth muscle cells 'downstream' from atherosclerotic lesions or sites of vascular damage (54).

We showed differences in the distribution of the receptors studied in different arteries in control animals and some significant differences between the control and diabetic groups.

It has been reported that in patients with diabetes, the ratio of vasodilator to vasoconstrictor substances in the vessel wall is shifted towards vasoconstriction (4, 55). Furthermore, it has been suggested that these altered responses and the distribution of these receptors could partially explain the presence of endothelial dysfunction (4, 55). This could explain the significant increase in the ET-1 and 5HT receptors in tissues of diabetic rabbits (although the 5HT difference was not significant); both of these receptors have potent vasoconstrictor actions. Similarly, the significant decrease of eNOS activity in tissue of diabetic rabbits contributed to vasoconstriction since NO is a potent vasodilator.

Furthermore, there is emerging evidence that the early use of statins may preserve renal function in patients with diabetes; this may occur possibly through NO and or ET-1-dependent actions (56-59). This possibility could be investigated using experimental models.

Conclusion

Significant differences were found in eNOS activity, as well as in the number of ET-1 and 5HT receptors in the aorta, renal and femoral arteries in control rabbits. Therefore, the "regional" receptor distribution varies according to the vessel being assessed. The number of ET-1 receptors was significantly higher in the aorta and arteries of diabetic rabbits compared with controls and the expression of eNOS was significantly reduced. The number of 5HT receptors was also higher in diabetic rabbits arteries but not significantly so.

Further studies need to identify the possibility of influencing these receptors and to evaluate their effect on pathology (*e.g.* re-stenosis and the distribution of arterial disease and calcification associated with diabetes).

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