L-Arginine–Nitric Oxide Pathway–Related Metabolites in the Aqueous Humor of Diabetic Patients

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PURPOSE. Nitric oxide (NO) is an important signal-transduction molecule that plays a significant role in the regulation of cardiovascular functions. In the L-arginine–NO pathway, NO synthase (NOS) converts L-arginine (L-Arg), the only known biologic substrate for NO formation, to NO and L-citrulline (L-Cit). Excessive NO production mediated by the inducible isoform of NOS has been implicated in the pathogenesis of various diseases. In the present study it was hypothesized that in vitreoretinal disorders such as diabetic retinopathy the production of L-Arg–NO pathway–related metabolites may be upregulated as a result of increased NO generation.

METHODS. From 20 eyes of nondiabetic subjects and 22 eyes of diabetic patients with ($n = 14$) and without ($n = 8$) diabetic retinopathy, undiluted samples of aqueous humor were drawn before cataract surgery. Levels of L-Arg, L-Cit, and the specific NOS by-product N^G-hydroxy-L-arginine (HOArg) were measured by high-performance liquid chromatography.

RESULTS. L-Arg, L-Cit, and HOArg were detected in all aqueous humor samples from diabetic and nondiabetic patients ($n = 42$). Comparison of HOArg levels in nondiabetic and diabetic subjects showed significantly higher levels in diabetic patients ($P = 0.002$). Concentrations of HOArg were higher in samples from patients with ($P = 0.005$) and without diabetic retinopathy ($P = 0.033$) than in control subjects. No statistically significant differences were observed in L-Arg or L-Cit levels.

CONCLUSIONS. Elevated levels of HOArg in the aqueous humor of diabetic patients reflect the possible role of NO as a significant factor in the regulation of retinal vascular functions and intraocular proliferative changes in diabetes mellitus in vivo. The control of intraocular NO production may constitute a potential therapeutic approach in diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2000;41:213–217)

Nitric oxide (NO) is an important intercellular signaling molecule that plays a prominent role in the vasodilatory responses of conduit and microcirculatory blood vessels. Results of both in vitro and in vivo studies suggest that NO is involved in the control of retinal blood flow under basal conditions and after retinal ischemia.[1] Furthermore, NO appears to contribute to the regulation of aqueous humor outflow.[2] In addition to its role as endothelium-derived relaxing factor, NO is also recognized as an antiproliferative and cytotoxic agent for many cell types.[3]

Situations in which high amounts of NO may be synthesized include inflammation, re-endothelialization, and angiogenesis. In these situations, the inducible isoform of nitric oxide synthase (iNOS), the enzyme that catalyzes the formation of NO from the terminal guanidino nitrogen of L-Arginine (L-Arg), is expressed after induction by cytokines or endotoxins.[4] Regarding angiogenesis, a process that is highly relevant to proliferative vitreoretinal disorders,[5] NO has been shown to be a potent inhibitor of cytokine-induced proliferation of endothelial cells.[6] In diabetic retinopathy, vascular damage in the retina may be mediated in part by NO.[7] Furthermore, NO has been shown to have an antiproliferative effect on retinal pigment epithelial cells in vitro, suggesting that the production of NO may contribute to the regulation of cell growth in proliferative vitreoretinal diseases.[3]

To study the biologic role of NO in a particular process requires measuring its concentration. Thus far, direct measurements of NO in vivo have been rare because of experimental difficulties and the short half-life of NO. In several experimental and clinical studies, NO synthesis has been monitored by the detection of nitrite (NO^-_2) and nitrate (NO^-_3) levels in biologic fluids. However, the concentration of these degradation products is partially attributable to sources other than NO synthase (NOS). In the quantification of NO production in vivo, the detection of L-Arg–NO pathway–related metabolites appears to be a reasonable approach. Because the conversion of L-Arg, the only known biologic substrate for NO formation, results in the formation of NO, L-citrulline (L-Cit) and, to some extent, a stable by-product, N^G-hydroxy-L-Arg (HOArg),[8–10] we hypothesized that the production of HOArg may be upregulated as a result of increased NOS activity in diabetic retinopathy. In the present study, we compared levels of L-Arg, L-Cit, and HOArg in the aqueous humor of diabetic and nondiabetic patients.

PATIENTS AND METHODS

Study Patients

Undiluted samples of aqueous humor were drawn by paracentesis from patients undergoing cataract surgery. The study was performed with the agreement of our ethics committee, and methods complied with the Declaration of Helsinki. We measured levels of L-Arg, L-Cit, and HOArg in 20 specimens of aqueous humor obtained from nondiabetic patients and in 22 specimens of aqueous humor obtained from diabetic patients with ($n = 14$) and without ($n = 8$) diabetic retinopathy. The patients' ages ranged from 50 to 82 years in the diabetic group and from 55 to 86 years in the nondiabetic group. The mean duration of diabetes by the time of intraocular surgery was 13.4 years (range, 6–27 years). Clinical data, including determinations of the stage of diabetic retinopathy, were obtained at the time of surgery with standardized forms. Of the 22 diabetic eyes, 14 had diabetic retinopathy. Of these, five had been subjected to conventional panretinal photocoagulation, and two had undergone additional previous cryotherapy and pars plana vitrectomy at least 1 year (range, 1–8 years) before the collection of aqueous humor samples. Seven patients showed only mild retinal changes consistent with nonproliferative diabetic retinopathy. The results in diabetic patients were compared with those in patients with no known underlying diabetes or potentially neovascularizing or proliferative disorder. Therefore, subjects with retinal vessel occlusion, rubeosis iridis, choroidal neovascular membranes, retinal detachment, uveitis, or retinitis were excluded from the control group. Moreover, we excluded patients from the study if they had evidence of glaucoma or any ocular or systemic inflammatory disease. Diabetic and nondiabetic subjects received prophylactic topical gentamicin exclusively before cataract surgery.

Sample Collection

Specimens were collected in sterile tubes and rapidly frozen at $-70°C$. The samples were then labeled anonymously without identifying clinical information and shipped on dry ice to the Center of Physiology and Pathophysiology at the University of Göttingen, Germany. Sample volumes ranged from 50 to 70 μl.

High-Performance Liquid Chromatography

Concentrations of L-Arg, L-Cit, and HOArg were measured by high-performance liquid chromatography–fluorescence detection analysis.[9] Analyses were performed with a low-pressure-gradient high-performance liquid chromatography pump (model L 6200 A; Merck–Hitachi, Darmstadt, Germany) and a fluorescence detector (model F1050, Merck, Darmstadt, Germany). Sample volumes of 10 μl were mixed with 10 μl *o*-pthaldialdehyde reagent at ambient temperature. The derivatized amino acids (5 μl per aliquot) were separated on a column (UltraTechsphere 5 ODS; HPLC Technology, Macclesfield, UK; 250 × 4.6 mm inside diameter) that was isocratically eluted with 10 mM potassium dihydrogenphosphate buffer (pH 5.85) acetonitrile-methanol-tetrahydrofuran (80:9.5:9.5:1, vol/vol/vol/vol). The flow rate was set to 1 ml/min, and the fluorescence of the eluate was continuously monitored at 425 nm (excitation, 338 nm). Levels of L-Arg, L-Cit, and HOArg were calculated on the basis of the integrated peak areas relative to those of authentic standards. After completion of each analysis, the column was washed with methanol for 15 minutes and

then re-equilibrated. The sensitivity of the high-performance liquid chromatography method for detection of the three amino acids was 0.02 to 0.04 μM. Because of the direct derivatization of the samples, both intra- and interassay variabilities were below 10%.

Statistical Analysis

Results are shown as medians and range. The significance of differences between corresponding groups of observations was evaluated by the Mann–Whitney test. The Spearman rank correlation coefficient was used to determine any correlation between patient age or duration of diabetes mellitus and levels of L-Arg, L-Cit, and HOArg. Acceptable significance was set at $P < 0.05$.

RESULTS

In our study, all samples of aqueous humor ($n = 42$) showed detectable concentrations of HOArg, L-Arg, and L-Cit. The levels of HOArg, L-Arg, and L-Cit from patients with diabetes mellitus were compared with those in specimens from patients without neovascular disorders (control subjects). The concentrations of HOArg in 22 samples from diabetic patients with or without retinopathy were significantly higher (median, 0.82 μM; range, 0.24–1.88 μM; $P = 0.002$) than those in 20 samples from the nondiabetic comparison group (median .0.4 μM; range, 0.04–0.64 μM).

The differences between the diabetic subgroup with retinopathy (median, 0.85 μM; range, 0.28–1.88 μM) and the diabetic subgroup without retinopathy (median, 0.78 μM; range, 0.24–1.24 μM) versus the control group were both statistically significant ($P = 0.0051$ and $P = 0.033$; Fig. 1). The differences among all three groups are illustrated in Figure 1. The concentrations of HOArg in samples from patients with quiescent neovascularization who had undergone previous cryotherapy and pars plana vitrectomy were 0.4 and 0.84 μM. Four of the seven samples from eyes with diabetic retinopathy that had the highest HOArg levels were from eyes that had undergone previous photocoagulation ($n = 3$) or additional vitrectomy ($n = 1$).

There was no correlation of HOArg concentrations with patient's age or duration of diabetes mellitus in any subgroup (data not shown).

Comparison of L-Arg levels in nondiabetic (median 12.52 μM; range 3.32–18.48 μM) and diabetic patients with and without retinopathy (median 8.88 μM; range 3.56–18.32 μM) showed no significant differences ($P = 0.36$; Fig. 2A; control subjects, $n = 18$ because of sample contamination). Moreover, in diabetic eyes (median, 7.12 μM; range, 0.44–15.2 μM) no difference in L-Cit levels compared with nondiabetic eyes (median, 6.76 μM; range, 2.76–10.48 μM) was noted ($P = 0.97$; Fig. 2B; control subjects, $n = 19$; diabetic patients without retinopathy, $n = 7$ because of sample contamination).

DISCUSSION

The formation of NO (and L-Cit) from L-Arg plays a major role in the regulation of cardiovascular functions. Under physiological conditions, the formation of NO involves a constitutively active calcium/calmodulin-dependent isoform of the enzyme NOS, eNOS, that is predominantly localized to the endothe-
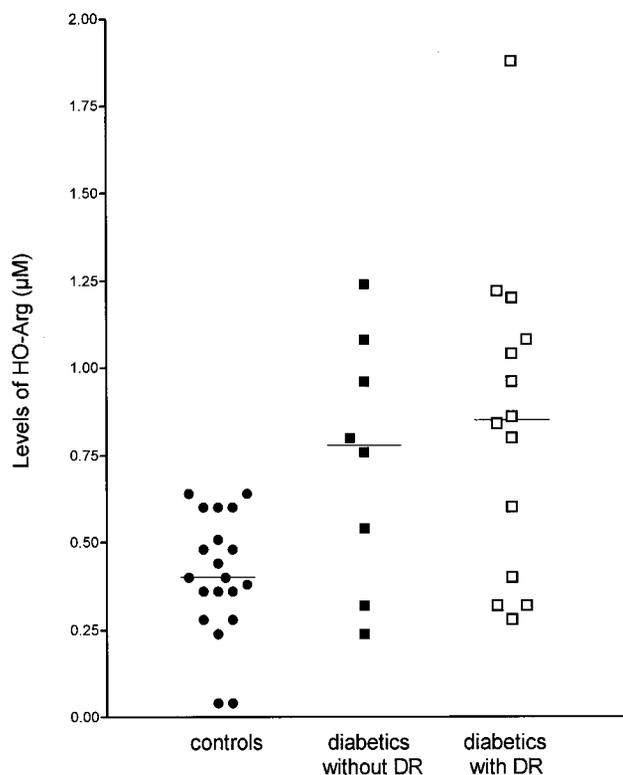
FIGURE 1. Levels of HOArg in aqueous humor of nondiabetic and diabetic subjects with and without diabetic retinopathy (DR), measured by high-performance liquid chromatography.

lium.[4] Moreover, the production of NO can be evoked by inflammatory mediators and cytokines because of the induction of a calcium-insensitive type of NOS, iNOS,[4] mainly in the vascular smooth muscle. In the course of NO formation, small amounts (2%–5% of the metabolized L-Arg) of a stable intermediate, HOArg, can be released from the active site of the enzyme in addition to NO and L-Cit.[8] Because NOS is the only known source of HOArg in the human body, HOArg constitutes a highly specific indicator for NOS activity and NO production in vivo. In our study, significantly elevated levels of HOArg were observed in the aqueous humor of diabetic patients. The release of substantial amounts of HOArg into the extracellular space has been shown in vivo and in vitro for various cell types expressing iNOS.[8] Once released from the NO-producing cell, HOArg competes with L-Arg for re-entry, most likely through the y^+ amino acid carrier, and usually accumulates in the extracellular space. HOArg uptake and metabolism may only be facilitated when the extracellular concentration of L-Arg decreases below a critical threshold.[8] Because HOArg production reflects only a small proportion of total NO activity, a significant increase in HOArg in the aqueous humor of diabetic patients suggests a considerable increase in NOS activity.

Interestingly, diabetic eyes with and without retinopathy contained comparable levels of HOArg, indicating that a maximally increased generation of NO occurs early in the course of diabetic retinal disease. However, there was no correlation of HOArg concentrations with duration of diabetes, although such an association is to be expected. From our data, it is not possible to state whether there is a relationship between the duration of diabetes mellitus and aqueous humor levels of
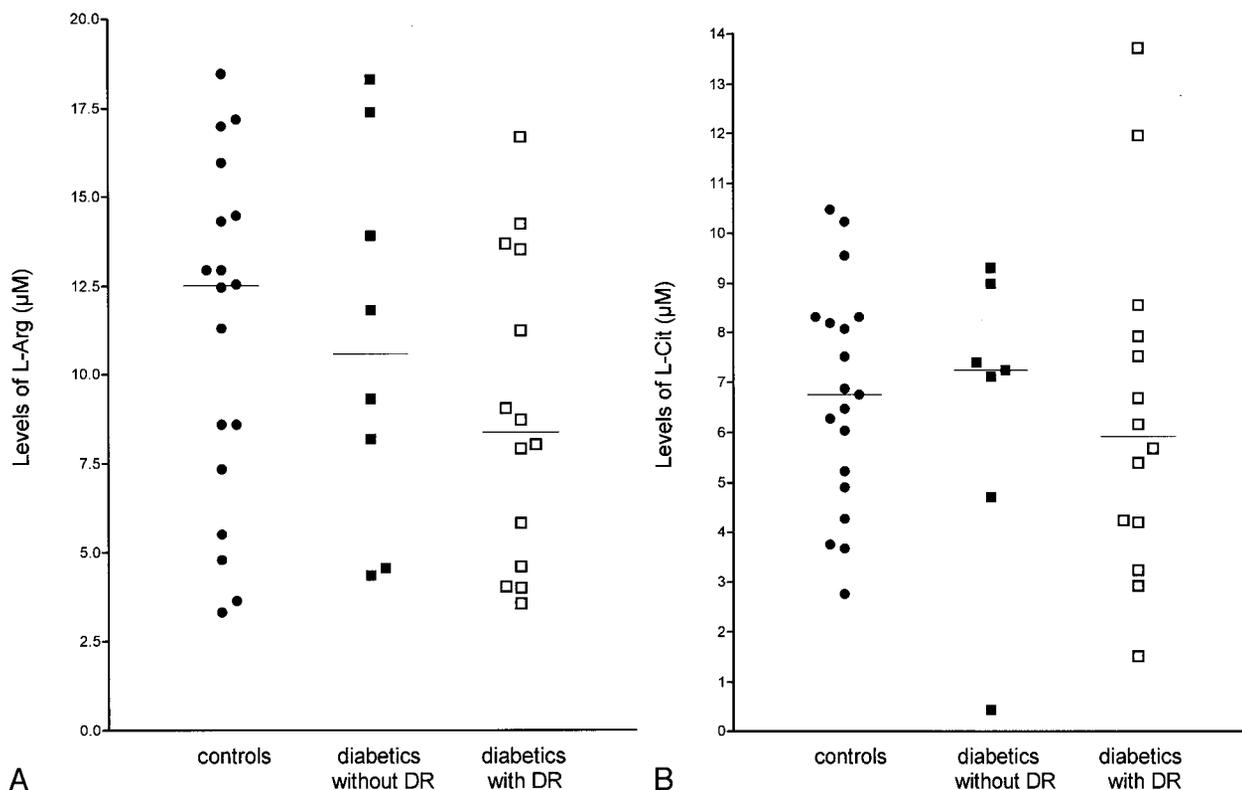

FIGURE 2. Levels of (**A**) L-Arg and (**B**) L-Cit in aqueous humor of nondiabetic and diabetic subjects, as determined by high-performance liquid chromatography.

HOArg, because all diabetic subjects had a history of ongoing diabetes mellitus for at least 6 years.

In the present study, we observed lower concentrations of L-Arg in the aqueous humor of diabetic patients than in nondiabetic patients, although this difference was not statistically significant. This is consistent with previous reports on NOS activity in vivo and may be in part the result of an increased uptake: Cellular sources of L-Arg include uptake of extracellular L-Arg, intracellular synthesis from L-Cit (recycling) as well as, to a lesser extent, intracellular proteolysis.[4,10] Moreover, L-Cit levels in diabetic and nondiabetic patients were not significantly different. The most likely explanation for this observation is that many NO-producing cells such as macrophages or endothelial cells can recycle L-Cit to L-Arg.[10] Because the availability of L-Arg constitutes a rate-limiting factor for NO production in vivo, the generation of L-Arg from L-Cit appears to be an important mechanism in cells with high NO activity, in particular in those cells expressing iNOS. Experimental studies have demonstrated that retinal vascular endothelial cells and pericytes synthesize iNOS under stimulated conditions, a mechanism that may be expected in diabetic retinal vascular disease.[11]

It is noteworthy that, under normal conditions, the vascular endothelium of the choroidal and anterior uveal circulations is likely to be the main source of NO in aqueous humor.[12] Because NO production has been shown to be lower in the diabetic vascular endothelium, it may be speculated that aqueous humor levels of NO metabolites are decreased in diabetic patients. However, it is not yet clear whether the bioavailability of NO (due to the increased formation of reactive oxygen species) rather than endothelial NOS activity or expression is responsible for the endothelial dysfunction associated with diabetes. Findings in studies of ocular hemodynamic reactivity to NO inhibition suggest that either NOS activity is increased or sensitivity to NO is decreased in patients with diabetes mellitus.[13]

The observation of enhanced NO production in diabetic eyes in vivo is consistent with reports on NO as a mediator of physiological and pathologic processes in the retina. It has been speculated that iNOS activity may be involved in diabetic retinal vascular damage through NO-induced increases in blood flow and vascular permeability. In diabetic rats, [^{125}I]-labeled albumin entry across the blood–retinal barrier, an indicator of altered vascular permeability, has been shown to be decreased by continuous administration of guanidine compounds.[7] Because guanidines inhibit iNOS, it may be hypothesized that iNOS activity is increased in the diabetic retina. However, because effects other than NOS inhibition may be mediated by these compounds, there is no definitive proof that NO produces diabetic retinal damage in vivo.

An increasing body of evidence that NO is an antiproliferative and cytotoxic agent for retinal cells has been provided in several experimental studies. Recent findings indicate that NO mediates neurotoxic actions of glutamate that are responsible for ischemic retinal injury.[14] In addition, glutamate neurotoxicity has been shown to be attenuated by the inhibition of NOS activity.[15] Other reports have demonstrated that bovine retinal pigmented epithelial cells express iNOS after activation with interferon-γ and lipopolysaccharide. It also has been shown that NO exhibits an antiproliferative action on RPE cells, whereas fibroblast growth factor has a protective effect by inhibiting the induction of NOS.[3]

From the current observations, it is not possible to implicate NO in the initiation of retinal damage in diabetic retinopathy, because factors promoting the expression of iNOS (and thus, enhanced NO formation) may precede its release into the aqueous humor. Because cytokines responsible for cellular proliferation and angiogenesis, such as vascular endothelial growth factor, basic fibroblast growth factor, or transforming growth factor-β_2 are found in vitreous and aqueous humor or in neovascular membranes from eyes with proliferative vitreoretinal disorders,[5,16] it is possible to suggest that persistently raised intraocular levels of NO may depend on a continuous operation of cytokine-mediated cellular reactions. Moreover, because NO has been shown to be a potent inhibitor of cytokine-induced proliferation of endothelial cells,[6] enhanced levels of NO-derived metabolites in aqueous humor may reflect a regulatory mechanism that is potentially relevant to ocular angiogenesis.[5] Therefore, we view the presence of high NOS activity in diabetic eyes as a reactive process.

Our observation that NO production appears to be increased in eyes of diabetic subjects without retinopathy supports the hypothesis that cytokine-mediated effects occur before the onset of morphologic changes in the vasculature. There is a reasonable likelihood that in pathologic situations such as diabetic mellitus in which the expression of iNOS is turned on, the generation of high concentrations of NO may be a significant factor in the regulation of retinal vascular functions and intraocular proliferative changes. The current findings merit further investigations to elucidate whether there is a direct relationship between the action of various cytokines and NO in vivo. An understanding of the endogenous inhibition of ocular cellular proliferation has a high degree of relevance, given the spectrum of potentially blinding complications in diabetic retinopathy.

Acknowledgments

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