

## Asymmetric Dimethylarginine (ADMA) in the Aqueous Humor of Diabetic Patients

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**Abstract.** Asymmetric dimethylarginine (ADMA) is an endogenous NO synthase (NOS) inhibitor whose production is enhanced by oxidative stress. Recent studies have shown that ADMA may also directly stimulate the production of reactive oxygen species (ROS) by up-regulation of the renin-angiotensin system independently of NOS inhibition. In this study, to investigate the clinical association of ADMA with diabetic retinopathy, we evaluated the levels of ADMA and NO oxides ( $\text{NO}_2^-$  and  $\text{NO}_3^-$ ) in serum and aqueous humor obtained during cataract surgery from non-diabetic subjects ( $n = 21$ ) and diabetic patients ( $n = 17$ ). We found that the ADMA existed in aqueous humor and its level was similar to that in serum. The ADMA levels in both serum and aqueous humor were higher in diabetic patients, especially those with severe retinopathy, than in the non-diabetic group (serum ADMA:  $0.67 \pm 0.26$  vs.  $0.53 \pm 0.08$   $\mu\text{mol/l}$ ,  $p < 0.05$ ; aqueous humor ADMA:  $0.55 \pm 0.20$  vs.  $0.32 \pm 0.16$   $\mu\text{mol/l}$ ,  $p < 0.05$ ). Also, the aqueous humor level of ADMA, but not the serum level, was correlated with HbA1c on analysis of all the patients ( $R = 0.33$ ,  $p < 0.05$  by simple regression analysis). However, a correlation between the ADMA levels in serum and aqueous humor was not observed in either the non-diabetic group or the diabetic group. Furthermore, serum and aqueous humor levels of NOx did not differ between the two groups, and no correlation with ADMA levels was observed in either group. These results suggest that ROS production may be enhanced in the eyes of diabetics. Since ADMA may act to potentiate ROS production independently of its inhibition of NOS, further investigation is required to clarify the possible contribution of ADMA to the development or progression of retinopathy.

**Key words:** Asymmetric dimethylarginine, Oxidative stress, Nitric oxide, Diabetic retinopathy, Aqueous humor

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**DIABETIC** retinopathy is a serious complication that leads to blindness among adults with diabetes. Diabetic retinopathy progresses from dysfunction of the retinal capillary endothelial cells and pericytes to simple and pre-proliferative retinopathy (SDR and PPDR) characterized by minimal hemorrhage and vessel occlusion. It then progresses to proliferative retinopathy

(PDR), characterized by the formation of new blood vessels on the retina. Although the exact mechanisms underlying the development and progression of retinopathy still remain unclear, previous studies have suggested that five processes induced by hyperglycemia may be linked to the etiology of diabetic vascular complications. These processes include activation of the polyol pathway, activation of the hexosamine pathway, excessive formation of advanced glycation endproducts (AGEs), activation of protein kinase C (PKC), and increased production of reactive oxygen species (ROS) [1, 2]. A recent study showed that these abnormalities are commonly associated with ROS production and that inhibition of ROS production could improve all of

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these abnormalities *in vitro* [3]. Thus, it may be important to investigate the cause of retinopathy by focusing on ROS. However, direct information has not been obtained regarding oxidative stress in the retinal tissue of diabetic patients.

Recently, asymmetric dimethylarginine (ADMA) has been attracting attention because of possible clinical implications as a new marker of ROS and also because of its possible pathophysiological effects. ADMA, symmetric dimethylarginine (SDMA), and N-monomethyl L-arginine (L-NMMA) are synthesized during the methylation of protein arginine residues by protein arginine methyltransferases (PRMT), and then are released during proteolysis. All three methylarginines are eliminated by renal excretion, but more than 90% of ADMA and L-NMMA is metabolized by dimethylarginine dimethylaminohydrolase (DDAH) to citruline and dimethylamine [4]. In experimental systems, accumulation of ADMA is accompanied by a decrease of DDAH activity despite a normal DDAH protein level, suggesting that the specific activity of the enzyme has declined [5]. Reduced sulfhydryl (-SH) groups of cysteine are essential for the activity of DDAH, making this enzyme extremely sensitive to oxidative stress [4]. Indeed, a decrease of DDAH activity is often accompanied by the accumulation of ROS, while antioxidants protect the enzyme [6, 7]. Oxidative stress may also stimulate the formation of ADMA, as evidenced by the increased expression of PRMT in endothelial cells subjected to high levels of ROS [6]. Thus, the serum ADMA level may be a marker of oxidative stress. Interestingly, ADMA and L-NMMA, but not SDMA, are endogenous competitive inhibitors of NO synthase (NOS) [8, 9]. However, the plasma concentration of L-NMMA is much lower than that of ADMA. Furthermore, ADMA may directly alter the activities of other arginine-metabolizing enzymes, such as arginine: glycine amidinotransferase or arginase [3], and may up-regulate the renin-angiotensin system via a mechanism unrelated to NOS blockade, since chronic infusion of ADMA induces microvascular lesions in wild-type and eNOS knockout mice [10]. Taken together, these findings indicate that ADMA may not only be a marker of increased ROS production, but may also be a toxic factor that causes vascular injury. Elevation of the plasma ADMA level has been detected in diabetic animals [5], but the role of ADMA in human diabetic microvascular complications has not been investigated. The ocular tissue may not only be

influenced by factors in the blood but also by those in aqueous humor, so that factors in both fluids may be linked with the development of retinopathy. However, it has not been examined whether ADMA exists in aqueous humor and whether it is associated with retinopathy. To investigate the association of ADMA with retinopathy, we examined ADMA and oxides of NO (nitrite and nitrate: NOx) in the serum and the aqueous humor of non-diabetic and diabetic subjects.

## Subjects and Methods

### Subjects

The subjects were 38 patients with or without diabetes who underwent cataract surgery from January to December 2005 at the Department of Ophthalmology of St. Marianna University Hospital (Kawasaki, Japan). They consisted of 17 diabetic patients with a mean age of  $66 \pm 11$  years (10 males and 7 females) and 21 non-diabetic subjects with a mean age of  $68 \pm 8.2$  years (7 males and 14 females). The diabetic patients were managed by diet alone ( $n = 5$ ), oral hypoglycemic agents ( $n = 7$ ), or insulin ( $n = 5$ ). Regarding diabetic complications, there were 12 patients with normal retinal finding or simple retinopathy and 5 patients with preproliferative or proliferative retinopathy. Among these 5 patients with severe retinopathy, 4 patients showed microalbuminuria but not overt proteinuria with abnormal elevation of serum creatinine level and 3 patients showed peripheral neuropathy.

Patients with smoking, liver disease, severe nephropathy (including those on end stage renal failure and/or dialysis), cancer, collagen diseases, infections, or a history of taking nitrates (which may affect NO production) were excluded from the study. All patients gave their written informed consent and the study was approved by the Ethics Committee of St. Marianna University School of Medicine (No. 877).

### Measurement of ADMA

During cataract surgery, approximately 100–300  $\mu$ l of aqueous humor was collected by an ophthalmologist via corneal puncture, transferred to sterile microtubes, and rapidly frozen at  $-80^{\circ}\text{C}$  for later analysis. The measurement of ADMA was performed as described previously with some modifications [9]. Coefficients

of variation (CV) of intra- and inter-assay in the HPLC system were 2.9% and 2.4%, respectively. On the day of measurement, EDTA-2Na containing serum or aqueous humor was mixed with 100% methanol at a 1 : 2 volume and centrifuged at 12,000 rpm for 15 min, after which 100  $\mu$ l of supernatant was injected into the HPLC apparatus (L-6200, L-7110; Hitachi, Tokyo, Japan). The samples were separated on ion exchange resin-packed column (NH2P-50: 4.6  $\phi$   $\times$  50 mm, Pegasil ODS: 4.6  $\phi$   $\times$  250 mm) with a column temperature of 47°C. Acid anhydride buffer was used as the mobile phase at a flow rate of 0.8 ml/min and step gradient elution was employed to extract ADMA. The ADMA level was calculated from the peaks detected in the wavelength range of 348–450 nm. Measurement was conducted by SRL, Inc. (Tokyo, Japan).

#### Measurement of NOx

Because NO has a very short half-life and thus is difficult to measure, we quantified its metabolic products (NOx), including nitrate ( $\text{NO}_2^-$ ) and nitrite ( $\text{NO}_3^-$ ). Sample preparation and storage of aqueous humor were done in the same manner as for the assay of ADMA. Then NOx was measured by the previously reported method with some modifications [11]. CV of intra- and inter-assay in the HPLC system were 1.4–6.4% and 2.1–6.7%, respectively. Serum or aqueous humor samples were injected into the HPLC apparatus (HPLC pump; L-7100, L-6000, Hitachi, Tokyo, Japan) for separation and reduction. Then the generated  $\text{NO}_2^-$  was reacted with naphthyl ethylene diamine, followed by detection at 540 nm using an SPD-10A detector (Shimadzu, Kyoto, Japan).

#### Statistical analysis

Results are expressed as the mean  $\pm$  SD. The significance of differences in mean values was analyzed by Student's t-test or one-way ANOVA, followed by Scheffe's multiple comparison test. To assess the relationship between ADMA levels in the serum or aqueous humor and clinical parameters, multiple regression analysis was performed.

## Results

The clinical profile of the subjects is shown in Table

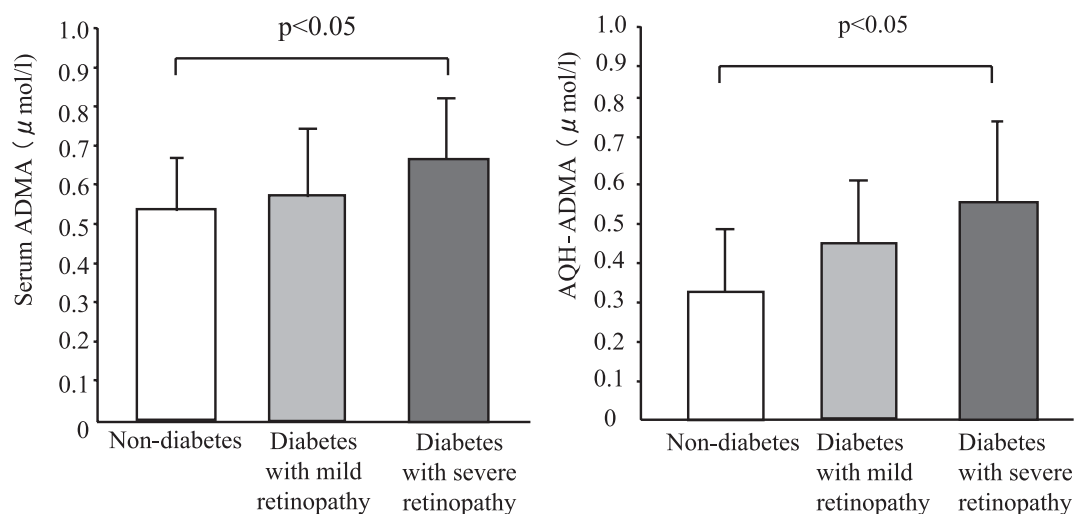
**Table 1.** Clinical characteristics of the subjects

	Diabetes	Non-diabetes
n (male/female)	17 (10/7)	21 (7/14)
Age (years)	65.8 $\pm$ 10.5	67.6 $\pm$ 8.2
Estimated duration of diabetes (years)	13.3 $\pm$ 9.7	
BMI ( $\text{kg}/\text{m}^2$ )	25.7 $\pm$ 3.2*	23.6 $\pm$ 3.8
HbA1c (%)	7.3 $\pm$ 1.2**	5.0 $\pm$ 0.3
T-CHO (mg/dl)	195 $\pm$ 33	206 $\pm$ 29
TG (mg/dl)	129 $\pm$ 72	115 $\pm$ 64
HDL-C (mg/dl)	49 $\pm$ 14*	58 $\pm$ 18
S-Cre (mg/dl)	0.7 $\pm$ 0.1	0.7 $\pm$ 0.2
SBP (mmHg)	136 $\pm$ 15*	128 $\pm$ 14
DBP (mmHg)	75 $\pm$ 5.8	73 $\pm$ 5.7

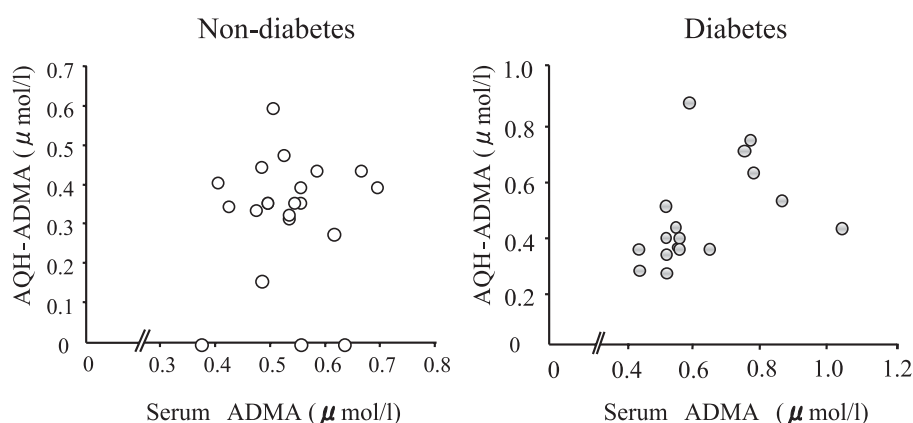
T-CHO: total cholesterol, TG: triglyceride, HDL-C: HDL-cholesterol, S-Cre: serum creatinine, SBP: systolic blood pressure, DBP: diastolic blood pressure, Mean  $\pm$  SD, \* $p$ <0.05 and \*\* $p$ <0.01 vs. Non-diabetes

1. While the mean age, mean total cholesterol level, mean triglyceride level, mean serum creatinine level, and mean diastolic blood pressure did not differ between the non-diabetic and diabetic groups, the mean HbA1c level, mean systolic blood pressure, and mean BMI were significantly higher and mean HDL-cholesterol was significantly lower in the diabetic group.

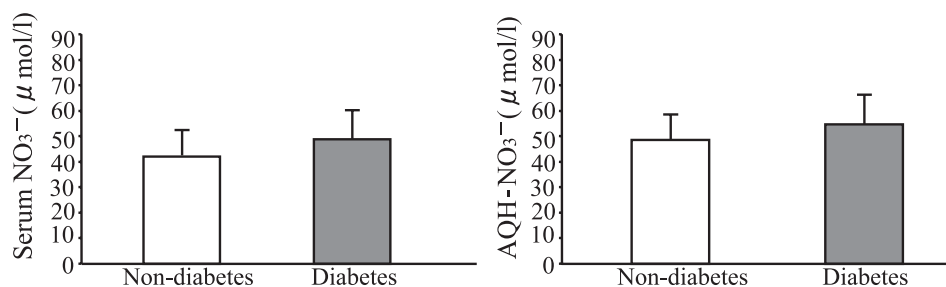
ADMA levels in both serum and aqueous humor were significantly higher in the diabetic group than in the non-diabetic group (serum:  $0.61 \pm 0.17$  vs.  $0.53 \pm 0.08$   $\mu\text{mol}/\text{l}$ ,  $p$ <0.05; aqueous humor:  $0.48 \pm 0.18$  vs.  $0.33 \pm 0.10$   $\mu\text{mol}/\text{l}$ ,  $p$ <0.05). The correlation between ADMA levels and clinical parameters as listed in Table 1 (gender, age, BMI, serum lipids, serum creatinine, and blood pressure) were evaluated by multiple regression analysis of data from all of the patients. Only HbA1c was positively correlated with the aqueous humor ADMA level (partial regression coefficient = 0.045, standard partial coefficient = 0.322,  $R = 0.346$ ,  $p = 0.036$ ), but no significant correlations with the serum ADMA level were observed. When the diabetic patients were further classified into a mild sub-group with no retinopathy or simple retinopathy ( $n = 12$ ) and a severe subgroup with pre-proliferative or proliferative retinopathy ( $n = 5$ ) by the Davis retinopathy classification, the serum ADMA levels and the adjusted aqueous humor ADMA values by HbA1c were significantly higher in the severe retinopathy subgroup than in the non-diabetic group (serum:  $0.67 \pm 0.26$  vs.  $0.53 \pm 0.08$   $\mu\text{mol}/\text{l}$ ,  $p$ <0.05; aqueous humor:  $0.55 \pm$



**Fig. 1.** Comparison of ADMA levels among non-diabetic subjects, diabetic patients with mild retinopathy or without retinopathy, and diabetic patients with severe retinopathy in serum and aqueous humor. Aqueous humor ADMA values are expressed as adjusted values by HbA1c. Data are expressed as the mean  $\pm$  SD.



**Fig. 2.** Correlation of serum and aqueous humor ADMA levels in the non-diabetic group and the diabetic group.



**Fig. 3.** Comparison of NOx levels between the non-diabetic group and the diabetic group in serum and aqueous humor. Data are expressed as the mean  $\pm$  SD.

0.20 vs.  $0.32 \pm 0.16$  μmol/l,  $p < 0.05$ ) as shown in Fig. 1. In the mild retinopathy subgroup, serum ADMA level and adjusted aqueous humor ADMA level by HbA1c showed intermediate values between those of

the severe retinopathy subgroup and the non-diabetic group, but no significant differences were observed (serum:  $0.58 \pm 0.11$  μmol/l; aqueous humor:  $0.46 \pm 0.16$  μmol/l). As shown in Fig. 2, there was no signifi-

cant correlation between serum and aqueous humor ADMA levels in the non-diabetic group ( $R = 0.08$ ,  $p = 0.75$ ) or the diabetic group ( $R = 0.42$ ,  $p = 0.09$ ).

As shown in Fig. 3, the NOx levels in serum and aqueous humor did not differ between the non-diabetic group and the diabetic group (serum NOx:  $42.2 \pm 23.2$  vs.  $49.1 \pm 29.4$   $\mu\text{mol/l}$ ; aqueous humor NOx:  $49.8 \pm 20.6$  vs.  $53.3 \pm 25.8$   $\mu\text{mol/l}$ ). Also, no significant differences of both serum and aqueous humor NOx levels were observed among the non-diabetic group, the diabetic subgroup with mild retinopathy, and the subgroup with severe retinopathy. Furthermore, there was no significant correlation between serum NOx and ADMA or between aqueous humor NOx and ADMA in both the non-diabetic and diabetic groups.

## Discussion

The present study revealed that ADMA could be detected in aqueous humor, where its concentration was similar to that in serum. It was also shown that the ADMA levels in serum and aqueous humor were higher in diabetic patients, especially those with severe retinopathy, than in non-diabetics, while the aqueous humor ADMA level, but not the serum ADMA level, was correlated with HbA1c on analysis of all the patients. In addition, there was no correlation between serum and aqueous humor ADMA levels in either the non-diabetic group or the diabetic group. Furthermore, serum and aqueous humor NOx levels did not differ between the two groups, and no correlation with ADMA was observed in either group.

We performed multiple regression analysis in all subjects, but not in each group separately, because the size of each group was not sufficient to analyze in the present study. Thus, we should confirm whether HbA1c level really affects aqueous ADMA content in non-diabetic subjects and diabetic patients by further large-sized studies. Since end-stage renal failure is one of the factors known to increase serum ADMA level [4], in the present study we excluded patients with severe nephropathy showing overt proteinuria and abnormal elevation of serum creatinine level. However, since neither the influence of end stage renal failure on aqueous ADMA content nor the association of early nephropathy with microalbuminuria or peripheral neuropathy with serum and aqueous ADMA levels has been evaluated, we also investigated these points.

The exact mechanism leading to elevation of the ADMA level has not been clarified, but decreased activity of DDAH, which metabolizes ADMA to citrulline and dimethylamine, has been implicated, because DDAH is inactivated by ROS [4]. DDAH has 2 isoforms, among which DDAH1 is predominantly expressed by tissues containing neuronal NOS (nNOS), and DDAH2 is mainly found in tissues containing endothelial NOS (eNOS) or inducible NOS (iNOS) [12, 13]. A previous study found the enhancement of ROS production by vascular endothelial cells under high glucose conditions, and showed that DDAH activity was significantly reduced [5]. In addition, incubation of human endothelial cells with polyethylene glycol-conjugated superoxide dismutase reversed the effect of high glucose on DDAH activity [5]. Although a direct investigation of DDAH2 in human vascular tissue has not been reported, hyperglycemia-induced enhancement of ROS production may possibly inactivate DDAH2 and thus increase the serum ADMA level. However, the serum ADMA level was not correlated with HbA1c in the present study, suggesting that a mechanism unrelated to hyperglycemia-unassociated mechanism may also exist.

Aqueous humor is secreted by the iris which consists of a ciliary body and vascular endothelial cells. It flows into the posterior chamber and then reaches the anterior chamber through the pupil. Next, it flows through the iridocorneal angle and the sinus venous sclerae, to finally enter the systemic circulation [14]. Aqueous humor is estimated to complete this cycle in about two hours [15], and the glucose concentration in the aqueous humor of the anterior chamber is almost the same as that in serum [16]. Thus, chronic hyperglycemia may increase ADMA production in the iris, and the positive correlation between HbA1c and the aqueous humor ADMA level seen in the present study supports this possibility. Interestingly, no correlation between serum and aqueous humor ADMA levels was observed in the diabetics, which suggests the possibility of intraocular ADMA production rather than simple transport of serum ADMA into the anterior chamber.

ADMA is a competitive inhibitor of all three NOS isoforms and its effect is reversed by a high concentration of L-arginine. It was previously found that ADMA inhibits NO production by isolated NOS and by intact cells with an  $\text{IC}_{50}$  of 2–10  $\mu\text{mol/l}$  [17], which is within the pathophysiological range. However, the NOx levels of serum and aqueous humor did not differ

between the non-diabetic and diabetic groups in the present study. Thus, although we did not evaluate the intracellular concentrations of L-arginine and ADMA, the elevated serum and aqueous humor ADMA levels in the diabetic group may not have been high enough to inhibit NO production *in vivo*. Apart from inhibition of NOS, ADMA has a NOS-independent action that up-regulates angiotensin-converting enzyme (ACE) expression and subsequent superoxide production via NAD(P)H oxidase located downstream of angiotensin II receptor 1 (AT1), as well as promoting vasoconstriction and vascular thickening in eNOS knock-out mice, with these abnormalities being suppressed by simultaneous treatment using an ACE-inhibitor or AT1 receptor blocker [10]. Therefore, ADMA itself may induce vascular lesions by increasing ROS production independently of its inhibition of NOS. There have been no reports regarding ADMA and eye disease, but

the present study showed that ADMA exists in the aqueous humor and its level was higher in diabetic patients. These results suggest enhanced oxidative stress in the eyes of diabetics, and raise the issue of whether ADMA is only a marker reflecting hyperglycemia-induced overproduction of ROS in ocular tissue or also contributes directly to the development or progression of retinopathy. Because the present findings were based on a small group of subjects, further *in vivo* and *in vitro* studies involve larger groups are needed to clarify the pathological significance of ADMA in diabetic retinopathy.

In conclusion, serum and aqueous humor levels of ADMA were higher in diabetics than in non-diabetics, and the aqueous humor ADMA level was correlated with HbA1c on analysis of all the patients. In contrast, serum and aqueous humor NOx levels did not differ between the two groups.

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