Zoology 114 (2011) 171–176

Contents lists available at ScienceDirect

Zoology

journal homepage: www.elsevier.de/zool

Naturally high plasma glucose levels in mourning doves (*Zenaida macroura*) do not lead to high levels of reactive oxygen species in the vasculature

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ARTICLE INFO

Article history:
Received 8 September 2010
Received in revised form 2 December 2010
Accepted 4 December 2010

Keywords:
Antioxidants
Birds
Oxidative stress
Mesenteric arteries
Resistance vasculature

ABSTRACT

Plasma glucose (P_{Glu}) concentrations in birds are 1.5–2 times higher than those of mammals of similar body mass. In mammals, sustained elevations of P_{Glu} lead to oxidative stress and free radical-mediated scavenging of endogenous vasodilators (e.g., nitric oxide), contributing to elevated blood pressure. Despite the relatively high P_{Glu} levels in birds, they appear resistant to the development of oxidative stress in tissues such as the heart, brain and kidneys. To our knowledge no information exists on oxidative stress susceptibility in the resistance vasculature of birds. Therefore, we compared endogenous antioxidant mechanisms in the resistance vasculature of mourning doves (MODO; *Zenaida macroura*) and rats (*Rattus norvegicus*). Reactive oxygen species (ROS) were assessed with the fluorescent indicator 7′-dichlorodihydrofluorescein diacetate, acetyl ester in mesenteric arteries from rats and wild-caught MODO. Despite having significantly higher P_{Glu} than rats, there were no significant differences in ROS levels between mesenteric arteries from rats or doves. Although superoxide dismutase and catalase activities were lower in the plasma, total antioxidant capacity, uric acid, vitamin E (α-tocopherol), and carotenoids (lutein and zeaxanthin) were significantly higher in MODO than in rats. Thus, compared to rats, MODO have multiple circulating antioxidants that may prevent the development of oxidative stress in the vasculature.

1. Introduction

Reactive oxygen species (ROS) are involved in a range of important signaling pathways within the vasculature and other tissues (Brownlee, 2001). However, in a hyperglycemic state, the electron transport chain becomes uncoupled, producing excessive ROS resulting in the development of oxidative stress (Brownlee, 2001; King and Loeken, 2004). Moreover, excess glucose interacts with proteins to form advanced glycation endproducts (AGE) (Yao and Brownlee, 2010), which can increase ROS production and create a vicious cycle (Farmer and Kennedy, 2009). ROS and AGEs can both cause tissue damage and lead to impaired vascular reactivity in mammals (King and Loeken, 2004).

Under normal conditions, mammals produce sufficient antioxidants to prevent ROS accumulation. An important antioxidant in mammals is superoxide dismutase (SOD), a potent scavenger of superoxide (O_2^-), which it converts to hydrogen peroxide (H_2O_2). Subsequently, catalase and glutathione peroxidase convert H_2O_2 to water and oxygen (Kyaw et al., 2004). In birds, uric acid is the main endogenous purine waste product (Wright, 1995). Previous studies of chickens (*Gallus gallus*) have demonstrated that inhibition of uric acid production results in increased oxidative stress and tissue aging (Klandorf et al., 2001; Simoyi et al., 2002), although this putative antioxidant role has not been examined in other avian species. Other sources of antioxidants in animals and humans include dietary vitamins (e.g., vitamin E) and carotenoids (Sies et al., 1992), although their role as antioxidants in birds is currently unclear.

Normal fasting plasma glucose (P_{Glu}) levels of birds are 1.5–2 times higher than those observed in mammals of similar body mass (Braun and Sweazea, 2008). In mammals, sustained elevations of P_{Glu} (hyperglycemia) lead to ROS production and eventual cell and tissue damage (Evans et al., 2002; King and Loeken, 2004). H_2O_2 and O_2^- are two notable ROS that are elevated in

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doi:10.1016/j.zool.2010.12.001

hyperglycemic mammals (King and Loeken, 2004), likely resulting from down-regulation of endogenous antioxidant pathways (West, 2000). Despite the naturally high P_{Glu} levels in birds, remarkable resistance to the formation of ROS has been documented in domestic pigeon (*Columba livia*) brain, heart and kidney tissues (Ku and Sohal, 1993).

Chronic oxidative stress has long been associated with decreased longevity in animals (Oliveira et al., 2010; Page et al., 2010; Salmon et al., 2010). However, many avian species live up to three times longer than mammals of similar body mass (Holmes et al., 2001). In prior studies, the mesenteric vascular bed of rats was shown to develop increased ROS in animals with even mild hyperglycemia induced by high fat feeding (Sweazea et al., 2010). The reasons for these apparent differences in the regulation of the tissue oxidative environment between mammals and birds are unclear. Therefore the objectives of this study were to: (i) compare the level of ROS in isolated mesenteric arteries from adult male mourning doves (MODO; *Zenaida macroura*) to the previously measured levels in adult male Sprague–Dawley rats (*Rattus norvegicus*) (Sweazea et al., 2010); (ii) compare plasma levels of glucose and activities of two common mammalian antioxidants, SOD and catalase, between rats and MODO; (iii) compare levels of other endogenous and dietary antioxidants: uric acid, α-tocopherol (vitamin E), and carotenoids (lutein and zeaxanthin) in the plasma of MODO and rats. We hypothesized that mourning doves have lower ROS production and higher levels of circulating antioxidants compared to rats, which may reduce or prevent hyperglycemia-induced oxidative stress in the vasculature.

2. Materials and methods

2.1. Experimental groups

Adult male mourning doves (MODO; *Z. macroura*) were captured the morning of each experiment from the Phoenix ($n = 7$) or Albuquerque metropolitan areas ($n = 5$) using walk-in style funnel traps that had been baited with wild birdseed. MODO were chosen as they are abundant in both Phoenix and Albuquerque and they are members of the same family (Columbidae) as the domestic pigeon (*C. livia*) in which research has been conducted on antioxidants and oxidative stress (Ku and Sohal, 1993). Moreover, previous studies have characterized glucose utilization by various tissues from MODO (Sweazea et al., 2006). Birds were transported to the laboratory in cloth bags or animal transport cages and blood was collected by venipuncture for the analysis of glucose, total antioxidant capacity, superoxide dismutase, catalase, uric acid, retinol, α-tocopherol, lutein and zeaxanthin. Animals were then euthanized by carbon dioxide asphyxiation, which was ensured by exsanguination secondary to removal of the mesenteric arteries. This is the approved method of euthanasia for birds at the University of New Mexico. The Arizona State University and the University of New Mexico Institutional Animal Care and Use Committees (IACUC) approved all protocols.

Male Sprague–Dawley rats (*R. norvegicus*) were purchased from Harlan Teklad (Madison, WI, USA) and housed at the animal care facilities at the University of New Mexico and the Arizona State University. Rats were fed a chow diet containing 57.33% kcal carbohydrates, 18% kcal protein, and 5% kcal fat, supplemented with vitamins A (15 IU/g) and E (110 IU/g) (formula 2018; Harlan Teklad, Madison, WI, USA). Rats were kept on a 12:12 h light:dark cycle and euthanized with sodium pentobarbital (200 mg/kg, i.p.), which is the approved method for rodents at the University of New Mexico. Blood samples obtained by cardiocentesis were collected at the University of New Mexico and the Arizona State University for analyses of glucose, total antioxidant capacity, superoxide dismutase, catalase, uric acid, retinol, α-tocopherol, lutein, and zeaxanthin.

2.2. Measurement of reactive oxygen species in isolated mesenteric arteries

Following euthanasia, a midline laparotomy was performed to expose and remove the mesenteric arcade of MODO. The arcade was immediately placed in ice-cold HEPES buffer (134.4 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose; pH 7.4 with NaOH), pinned out in a Silastic-coated dissection dish, and fifth-order mesenteric resistance arterioles (~1 mm length; 80–120 μm inner diameter) were isolated. Isolated arterioles were then transferred to a vessel chamber (CH-1; Living Systems Instrumentation, Burlington, VT, USA) filled with HEPES, cannulated with glass pipettes, and secured in place with silk ligature. The vessels were then stretched longitudinally to approximate *in situ* length, pressurized to 60 mm Hg with a servo-controlled peristaltic pump (Living Systems Instrumentation, Burlington, VT, USA), and the chamber placed on a microscope stage for analysis. Blood vessels were superfused with warm (41 °C, avian normal body temperature) avian physiological salt solution (APSS; 144 mM NaCl, 25 mM $NaHCO_3$, 10 mM $NaC_2H_3O_2$, 5.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 0.50 mM $MgCl_2$, and 11.1 mM glucose; pH 7.4), which has been used in prior studies of isolated avian vessels (Yamaguchi and Nishimura, 1988; Stallone et al., 1990). This solution was aerated with 21% O_2, 6% CO_2, and balance N_2 gas mixture throughout the experiments to maintain pH and provide adequate oxygenation of the isolated blood vessels.

Vessel chambers were transferred to a Nikon Diaphot 300 microscope equipped with a 10× fluorescence (FITC) objective for analysis (Nikon Corp., Tokyo, Japan). After the vessels were allowed to equilibrate for 30 min in a control APSS solution, arteries were loaded in the dark with the cell-permeant ROS-sensitive fluorescence indicator, 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester (DCF; Molecular Probes, Eugene, OR, USA) in a vessel chamber attached to a temperature controller (Living Systems Instrumentation, Burlington, VT, USA). The temperature of the chamber was maintained at physiological levels for MODO (41 °C).

DCF is oxidized by cytoplasmic peroxynitrate ($ONOO^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (^-OH) to produce a fluorescent product (Crow, 1997; Myhre et al., 2003; Keller et al., 2004). Images were collected before DCF loading, for background, and 50 min later using a cooled, digital CCD camera (SenSys 1400; Photometrics, Tucson, AZ, USA). MetaFluor 4.5 software (Universal Imaging, Downingtown, PA, USA) was used for processing images.

Specificity of DCF fluorescence as a measure of ROS was verified in prior experiments (Jernigan et al., 2004; Sweazea and Walker, 2009; Sweazea et al., 2010). In these prior studies, the levels of DCF fluorescence in isolated mesenteric arteries were measured for male Sprague–Dawley rats (Sweazea et al., 2010). These data are shown in Fig. 2.

2.3. Measurement of plasma glucose, total antioxidants, superoxide dismutase, catalase, and uric acid

P_{Glu} levels were measured using a glucose oxidase kit (Cat. 10009582, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol.

The total antioxidant capacity of the plasma from rats and MODO was measured using an antioxidant assay kit (Cat. 709001; Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. In this assay, antioxidants present in the plasma

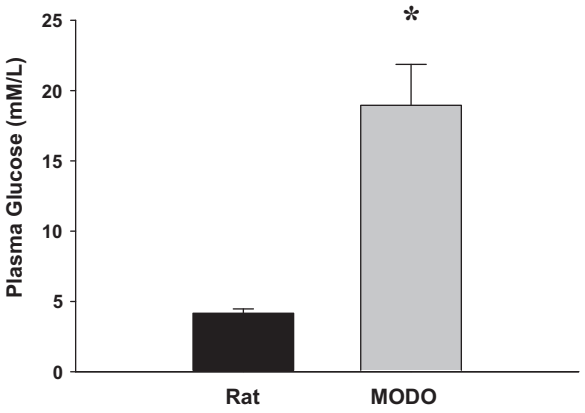

Fig. 1. Plasma glucose levels in rats ($n = 6$) and mourning doves (MODO; $n = 5$). Data are expressed as mean ± SEM; $^*p < 0.05$ vs. rats.

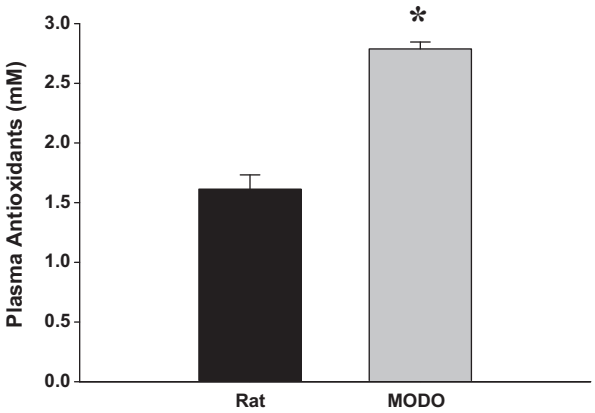

Fig. 3. Plasma total antioxidant capacity in rats ($n = 6$) and mourning doves (MODO: $n = 5$). Data are expressed as mean ± SEM; $^*p < 0.001$ vs. rats.

inhibit the oxidation of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) by metmyoglobin. The amount of oxidized ABTS produced is then measured spectrophotometrically at 750 nm.

A commercially available kit (Cat. 706002, Cayman Chemical, Ann Arbor, MI, USA) was used to measure plasma superoxide dismutase activity according to the manufacturer's protocol. The kit utilizes the interaction between hypoxanthine and xanthine oxidase to produce O_2^- radicals, which are detected using tetrazolium salt. The kit detects all three types of SOD metalloenzymes: copper/zinc, manganese, and iron. One unit of SOD is defined by the manufacturer as the amount of enzyme required to dismutate 50% of the O_2^- radicals.

Plasma catalase activity was measured using a commercially available kit (Cat. 707002; Cayman Chemical, Ann Arbor, MI, USA). In this assay, catalase reacts with methanol in the presence of H_2O_2 to produce formaldehyde, which is then measured by adding 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole to form a purple reaction product that can be detected spectrophotometrically.

Plasma uric acid levels were measured with the QuantiChrom Uric Acid Assay Kit (Cat. DIUA-250; BioAssay Systems, Hayward, CA, USA) that utilizes 2,4,6-tripyridyl-s-triazine, which forms a blue complex with iron in the presence of uric acid.

For each assay, all samples were run in duplicate on one assay plate to eliminate inter-assay variations.

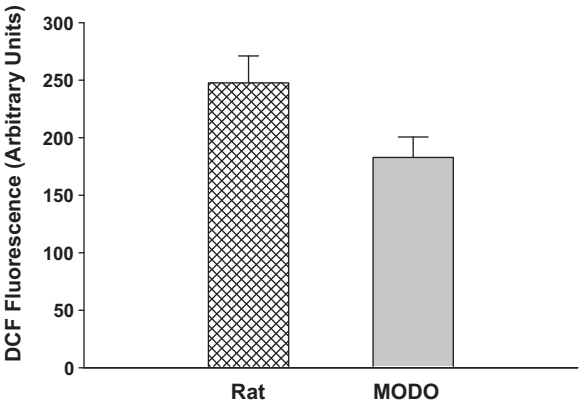

Fig. 2. DCF (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) measurement of radical oxygen species in isolated mesenteric arteries from rats ($n = 5$) and mourning doves (MODO; $n = 5$). Data for rats obtained from a previous study by the authors (Sweazea et al., 2010). Data are expressed as mean ± SEM and are not significantly different ($p = 0.093$) between rats and doves.

2.4. Measurement of plasma vitamins A and E as well as carotenoids

Carotenoids as well as vitamins A and E were extracted from a 30 μl sample of plasma from each MODO by adding 300 μl of ethanol, vortexing, then adding 300 μl of a 1:1 mixture of *tert*-butyl methyl ether and hexane, vortexing, and finally centrifuging at 10,000 rpm for 2 min. For the rat plasma the same procedure was followed, but 100 μl of rat plasma in 500 μl of ethanol and 500 μl of *tert*-butyl methyl ether and hexane were used for the extraction. These conditions have been shown to repeatably recover common carotenoids in plasma (McGraw et al., 2008). The supernatant from each extraction was then collected, evaporated to dryness under a stream of nitrogen, and resuspended in 200 μl of the mobile phase (42:42:16 by volume, methanol:acetonitrile:dichloromethane). For high performance liquid chromatography (HPLC) analysis, 50 μl of the resuspended extract was injected into a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) fitted with a Waters YMC carotenoid 5 μm column (4.6 mm × 250 mm) with the column heated to 30 °C. A three-step gradient solvent system was used as described in McGraw et al. (2006) to separate and quantify xanthophylls and carotenes. Absorbance data were collected from 300 to 550 nm using a Waters 2996 photodiode array detector, and carotenoids and vitamins were identified by comparing their retention times and absorbance maxima (λ_{max}) to those of external standards.

2.5. Statistical analyses

All data were analyzed by one-way ANOVA to compare differences between doves and rats. Where effects were significant, individual groups were compared using Student–Newman–Keuls post hoc analysis for comparisons with the same number of samples per group or Dunn's post hoc analysis for uneven sample sizes between groups. A probability of ≤ 0.05 was accepted as statistically significant for all comparisons.

3. Results

3.1. Plasma glucose

Mourning doves have significantly higher P_{Glu} concentrations (18.9 ± 2.91 mM/l) than rats (4.2 ± 0.31 mM/l; Fig. 1), which is consistent with earlier observations (Braun and Sweazea, 2008) and would be expected to produce significant oxidative stress in the vasculature.

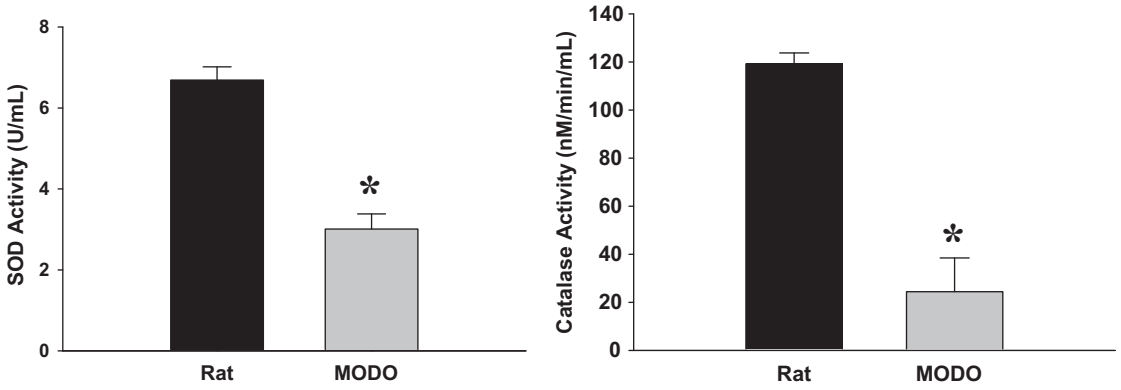

Fig. 4. Plasma superoxide dismutase (SOD) and catalase activity levels in rats ($n = 6$) and mourning doves (MODO; $n = 5$). Data are expressed as mean ± SEM; $^*p < 0.005$ vs. rats.

3.2. Measurement of DCF fluorescence

Despite the relatively high P_{Glu} in MODO, isolated mesenteric arteries showed similar levels of ROS compared to our recently published levels in rats measured in the same laboratory (Sweazea et al., 2010; Fig. 2). These findings suggest that MODO arteries are protected from hyperglycemia-mediated increases in ROS.

3.3. Plasma total antioxidant capacity

Data in Fig. 3 show that plasma total antioxidant capacity was significantly greater in birds (2.8 ± 0.06 mM/l) than in rats (1.6 ± 0.12 mM/l), supporting the hypothesis that MODO have higher levels of circulating antioxidants than rats to protect their vasculature from the development of glucose-mediated oxidative stress. The assay for total antioxidant capacity does not distinguish between specific antioxidants, but rather measures the combined activities of all circulating antioxidants. Therefore, common antioxidants that were predicted to be present in birds were measured.

3.4. Plasma superoxide dismutase, catalase, uric acid, vitamins and carotenoids

Superoxide dismutase (SOD) and catalase activities (Fig. 4) were significantly lower in MODO plasma (3.0 ± 0.37 U/ml, 24.4 ± 14.1 nM/min/ml, respectively) compared to rats (6.7 ± 0.33 U/ml, 119.2 ± 4.55 nM/min/ml, respectively). These findings suggest that SOD and catalase play a minimal role in the regulation of ROS in the vasculature of healthy birds but may be upregulated under conditions of oxidative stress. In contrast, data in Fig. 5 show that MODO have higher circulating levels of uric acid (8.2 ± 0.87 mg/dl) than rats (1.8 ± 0.18 mg/dl). Moreover, the dietary carotenoids lutein and zeaxanthin were found in significant abundance in MODO plasma (2.6 ± 0.31 μg/ml lutein; 1.2 ± 0.13 μg/ml zeaxanthin) when compared to rat plasma where these carotenoids were undetectable. The circulating levels of vitamin E (α-tocopherol; Fig. 6) were likewise significantly higher in MODO plasma (25.6 ± 2.54 μg/ml) than in rat (11.0 ± 0.50 μg/ml). Since some carotenoids are precursors to the formation of vitamin A (retinol), levels of retinol were measured (Fig. 6) and found to be at significantly higher levels in MODO plasma (4.1 ± 0.05 μg/ml) than in rats (1.4 ± 0.02 μg/ml).

4. Discussion

High P_{Glu} levels in mammals are strongly associated with the development of oxidative stress (West, 2000; Evans et al., 2002; Wiernsperger, 2003; Johansen et al., 2005). In contrast, despite their naturally high P_{Glu} level (Fig. 1), levels of ROS in isolated mesenteric arteries of MODO were similar to previously measured levels in rats (Fig. 2; Sweazea et al., 2010). These findings are in contrast to other tissues (brain, heart and kidney) of the domestic pigeon (*C. livia*), which show significantly lower levels of ROS production by isolated mitochondria than those found in rats (Ku and Sohal, 1993). These discrepancies may reflect the varying needs for ROS in the normal cellular activities of each tissue (artery vs. brain, heart and kidney). This has been shown in mammals where, under normal conditions, ROS play an important regulatory role in many physiological processes (Oliveira et al., 2010; Salmon et al., 2010). In fact, our group has demonstrated a physiological role for H_2O_2 in mediating vasodilation in rats (Sweazea et al., 2010). The rat physiological salt solution used in the prior studies contained 5.5 mM glucose, which is the approximate physiological level of glucose for this species (Sweazea et al., 2010). In contrast, the avian physiological salt solution contained 11.1 mM glucose. Despite having twice the amount of glucose in the superfusate, the level of vascular ROS was similar between both species, reflecting either higher antioxidants or lower ROS production in doves. Therefore, the similar vascular levels of ROS in rats and birds (Fig. 2) may indicate an important role for ROS in the maintenance of normal vascular reactivity of birds, with levels closely regulated by endogenous and dietary antioxidants. Alternatively, the lower levels in doves may reflect a lower production of ROS.

Since rats do not have such high P_{Glu} levels, their need for antioxidants is expected to be much lower than that of birds. Therefore, the hypothesis was that ROS production is lower and levels of circulating antioxidants are higher in birds than in rats to protect them from the development of vascular oxidative stress. In sup-

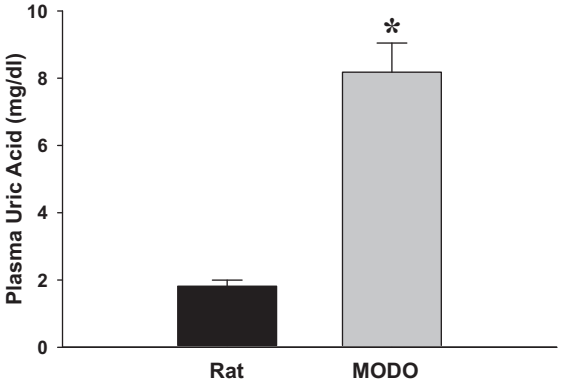

Fig. 5. Plasma uric acid concentrations in rats ($n = 6$) and mourning doves (MODO; $n = 5$); $^*p < 0.01$ vs. rats.

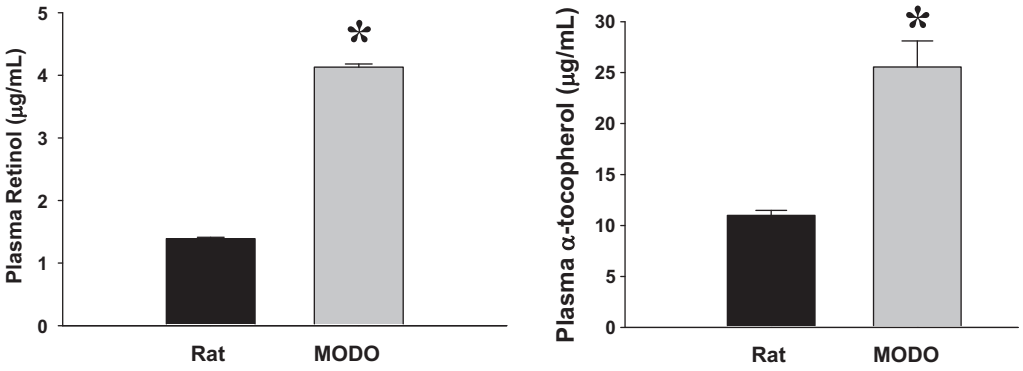

Fig. 6. Plasma retinol (vitamin A) and α-tocopherol (vitamin E) concentrations in rats ($n = 6$) and mourning doves (MODO; $n = 7$); $^*p < 0.005$ vs. rats.

port of this hypothesis, the circulating total antioxidant capacity of MODO was significantly greater than that of rats (Fig. 3). Since this assay did not distinguish between antioxidants, the levels of specific antioxidants were measured individually. As shown in Fig. 4, the antioxidant activities of SOD and catalase were significantly less in MODO than in rats. These data suggest that SOD and catalase have minimal roles in the regulation of vascular ROS in healthy MODO. This is in contrast to healthy rats in which both SOD and catalase play major roles in the regulation of vascular ROS and the maintenance of normal vascular reactivity (Lynch et al., 1997; Laight et al., 1998; Sweazea et al., 2010). It is possible that increased activities of both SOD and catalase may be observed under pathological conditions of augmented ROS production in MODO, although this was not examined in the present study.

Uric acid is the main endogenous purine waste product in birds (Wright, 1995) and is a powerful antioxidant (Machin et al., 2004). In fact, it has been previously shown to account for as much as 90% of the variation in total antioxidant capacity measured in wild birds (Cohen et al., 2007). Its role as an antioxidant in chickens (*G. gallus*) is evidenced by increased oxidative stress and tissue aging following administration of the uric acid inhibitor allopurinol (Klandorf et al., 2001; Simoyi et al., 2002). MODO have significantly greater circulating levels of this antioxidant compared to rats (Fig. 5), which may help to protect the vasculature from increases in ROS. Uric acid is also the most abundant circulating antioxidant in humans (Peden et al., 1990; Maxwell et al., 1997; Waring, 2002; Waring et al., 2003). In humans, however, it has been shown to play a dual pro-oxidant role that would promote oxidative stress (Sanguinetti et al., 2004; So and Thorens, 2010). Moreover, in high concentrations uric acid can lead to the formation of urate crystals that cause gout in humans (So and Thorens, 2010), and hyperuricemia has been linked with impaired vasodilation of blood vessels, which can contribute to cardiovascular disease (Corry and Tuck, 2006). Although not common, gout has been reported in birds including ratites, raptors and psittacines (Rothschild and Ruhli, 2007), and can be induced in chickens (*G. gallus*) by feeding a diet high in calcium and protein (Guo et al., 2005).

Dietary vitamins and carotenoids also provide a rich source of antioxidants for birds. Vitamin E (α-tocopherol) belongs to a class of lipid-soluble tocochromanols that are essential to the normal health of animals (Hidiroglou et al., 1992). Since it is synthesized in plants, it must be obtained through the diet (Falk and Munne-Bosch, 2010). It has long been known to have antioxidant properties (Mezes et al., 1997; Colombo, 2010) and was found to be circulating at significantly higher levels in the plasma of MODO than in rats (Fig. 6) despite the supplementation of the rat diet with vitamin E. The dietary carotenoids lutein and zeaxanthin were also present at high concentrations in the plasma of MODO but were undetectable in rats (see Section 3.4). Some carotenoids are also precursors to vitamin A (retinol), which was found to be circulating at higher levels in MODO than in rats (Fig. 6) despite the supplementation of the rat diet with vitamin A. Retinol is essential to normal reproductive capacity and vision as well as the maintenance of healthy epithelia (Rogers, 1994).

In conclusion, MODO have a variety of endogenous and dietary antioxidant mechanisms that limit oxidative stress and tissue damage that would typically be observed in mammals with such high plasma glucose concentrations. Since similar levels of ROS in rats contribute to normal vascular reactivity, examination of the potential physiological role of ROS in vascular control in avian arteries is warranted. An alternative hypothesis for explaining avian tolerance of naturally high plasma glucose levels has been raised by Hargrove (2005), who postulated that avian red blood cells may be resistant to glycation because of their high turnover rate (~21 days) in comparison to mammals (~120 days for humans). If other proteins likewise exhibit a high turnover rate, then this could certainly contribute to the lower levels of protein glycation and oxidative stress observed in birds.

Acknowledgements

The authors would like to thank Lori Hurley, Arizona State University, for collecting blood from MODO by jugular venipuncture. All birds were collected under scientific collecting permits from the US Fish and Wildlife Service (B.O.W.: MB094297-0; K.L.S.: MB202760-0) and New Mexico (B.O.W.: 3217) as well as Arizona Game and Fish (K.L.S.: SP591031) Departments. This work was supported by HL63207 from the National Heart, Lung, and Blood Institute (to B.R.W.) and new faculty start-up funds provided by Arizona State University (to K.L.S.).

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