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Expression of thromboxane synthase and the thromboxane-prostanoid receptor in the mouse and rat retina

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Abstract

Experimental models of the diabetic retina have suggested a pathological role for thromboxane. To date however, little information is available as to the cellular locations of retinal thromboxane synthase (TxS), or its receptor, even in non-diabetic controls. In this study, C57BL/6 mice and Wistar rats were injected with streptozotocin to induce diabetes, or with buffer for non-diabetic controls. Four weeks following the injection, eyes were enucleated and labeled for TxS and the thromboxane-prostanoid (TP) receptor. Immunofluorescent intensity was quantified in the ganglion cell plus inner plexiform layers, inner nuclear layer, outer plexiform layer, outer nuclear layer, and photoreceptor inner segment. Even in control mice and rats, all layers of the retina showed immunoreactivity for TxS and the TP receptor: however, the pattern of expression demonstrated an inverse relationship, with the highest TxS staining in the inner retina, and the highest TP receptor staining in the outer retina (more specifically, in the photoreceptor inner segment). Four weeks of hyperglycemia did not increase the retinal levels of TxS or TP receptor; however, TP receptor intensities in the outer retina of diabetic rats were highly variable (mostly high but some low), with no values from the photoreceptor inner segment in the same range as obtained from controls.

Keywords

Thromboxane Synthase; Thromboxane-Prostanoid Receptor; Streptozotocin; Mouse; Rat; Diabetes; Immunofluorescence

Introduction

Diabetic retinopathy (DR) continues to be a leading cause of new cases of blindness in adults (Fong et al., 2004). The pathogenesis of DR is multifaceted and the mechanisms involved in the development of DR have not been fully elucidated (Cai and Boulton, M., 2002; King et al., 1996). The pathways leading to vision loss may include a role for the prostanoid thromboxane A₂ (TxA₂), which has been found to mediate various pathological events such as platelet aggregation, vasoconstriction, and inflammation. Platelet TxA₂, as measured by the stable metabolite TxB₂, has been reported to increase in experimental diabetes (De La Cruz et al.,

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1998; De La Cruz et al., 2000; Moreno et al., 1995). However, platelets are not the only cells that can produce TxA₂, which may be released locally by other cells in the retina.

In most tissues throughout the body, arachidonic acid is broken down to prostaglandin H₂ (PGH₂) via the cyclooxygenase enzyme. In cells that produce thromboxane synthase, PGH₂ can be converted to TxA₂, which is rapidly and spontaneously converted to the inactive molecule thromboxane B₂. TxA₂ binds to the thromboxane-prostanoid (TP) receptor, of which there are two isoforms: TP α and TP β (Ullrich et al., 2001).

Experimental evidence from the rat suggests a role for thromboxane in the pathology of the diabetic retina. In as little as two weeks following streptozotocin-induced hyperglycemia (Moreno et al., 1995), platelet production of TxA₂ was found to be elevated, and retinal vascular density found to be attenuated, with these consequences progressing in magnitude in the following weeks. The decrease in retinal vasculature (< 70% reduction by 90 days) was almost completely restored with daily treatment of a dual thromboxane synthase inhibitor / receptor antagonist (De La Cruz et al., 2000), or with a thromboxane synthase inhibitor alone (De La Cruz et al., 1998). The thromboxane-dependent decreases in capillary density potentially could be related to the ability of the prostanoid to constrict vascular smooth muscle, and indeed, our own lab has found an attenuation of retinal arteriolar constriction in diabetic animals (hyperglycemic for 3–4 weeks) by targeting the TxA₂ pathway (Lee et al., 2008; Lee and Harris, N. R., 2008; Wright et al., 2009; Wright and Harris, N. R., 2008). However, thromboxane may have widespread effects throughout the retina beyond that of vasoconstriction, and in fact, the TP receptor has been found in the photoreceptors of the human retina (Chen et al., 1994). However, to our knowledge, the retinal locations of the receptor and synthase have yet to be described in mice or rats: nor is it known whether diabetes induces any change in this distribution.

Therefore, the aims of the present study were to 1) determine the location of thromboxane synthase and thromboxane-prostanoid receptors in the retinas of mice and rats, and 2) determine whether levels of these molecules are altered by four weeks of hyperglycemia in the streptozotocin model of diabetes.

Materials and methods

2.1. Animals

Male C57BL/6 mice (Jackson Laboratories) weighing 24–29 grams and male Wistar rats (Harlan Laboratories) weighing 129–164 grams were randomly assigned to intraperitoneal (i.p.) injection of streptozotocin (STZ; Sigma, St. Louis, MO) dissolved in pH 4.5 sodium citrate buffer, or injection of sodium citrate buffer alone. STZ was injected into the animals (180 mg/kg to mice; 60 mg/kg to rats) within 15 min of preparation. Non-fasting blood glucose levels were checked six days following STZ injection via a tail vein puncture and again on the day of the experiment using a One Touch Ultra Glucometer (Milpitas, CA). Mice and rats remained on the protocol for four weeks, and the diabetic animals were included in the study if glucose values on day six and on the day of the experiment exceeded 250 mg/dl. However, two rats were included in the study despite our not obtaining a glucose level on day six following STZ injection, but with the rats having glucose values > 550 mg/dl on the day of the experiment. Body weight was recorded three times per week beginning on day six following STZ injection. Mice and rats received 0–2 units insulin/kg of body weight, (Humulin R; Eli Lilly & Co., Indianapolis, IN) 0–3 times per week as needed to partially attenuate any measured loss in body weight. At least 48 hours prior to the experiment, insulin was stopped. Mice and rats were housed one per cage and received water and standard chow. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Immunofluorescence Labeling

On the day of the experiment, mice were euthanized with an overdose of Nembutal sodium solution (pentobarbital) and rats were euthanized with Enflurane. The right eye was immediately enucleated and placed in phosphate buffered 4% paraformaldehyde (FD Neurotechnologies, Inc., Baltimore, MD) for 2.5 hours. The anterior portion of the eye and lens were removed and the eyecup (retina, choroid, and sclera) was placed in 15–20% sucrose solution at 4° C overnight for cryoprotection (Chen and Nathans, J., 2007; Nishiguchi et al., 2008). Eyecups were placed in optimal cutting temperature (OCT) compound and cut at 10 μ m thickness.

Tissue sections on slides were washed for 5 min (twice) in Triton X-100 with gentle agitation then incubated in goat serum for 1.5 hours. Slides were then incubated with primary antibody mixed in PBS + 1% bovine serum albumin (BSA) overnight at 4° C. The next day, slides were washed twice with Triton X-100 for 5 min with gentle agitation followed by incubation with secondary antibody mixed in PBS + 1% BSA for 1 hour in the dark. Slides were washed twice in PBS for 10 min and mounted with Vectashield® Hardset™ Mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). Negative control slides were labeled following the same procedures as test slides, but without the primary antibody in the PBS + 1% bovine serum albumin (BSA).

Images were collected using a CoolSNAP ES camera (Photometrics, Tucson, AZ) attached to a Nikon Eclipse E600FN microscope with a Gibraltar™ Burleigh stand and an X-Cite™ 120 fluorescence illumination system as a light source. Images were obtained using a 4x objective with a Nikon FITC filter and an auto exposure time of 500 ms. For each image of immunostaining collected with the FITC filter, a counterstained image (DAPI-stained nuclei) was also obtained. Light intensity measurements were taken using a radiometer photometer (model ILT 1400-A; International Light Technologies; Peabody, MA) before and after each set of images were collected from one slide. NIS Elements Basic Research software version 3.0 (Nikon Instruments Inc., Melville, NY) was used for capturing and analyzing data.

Thromboxane synthase was labeled with a rabbit polyclonal antibody against thromboxane synthase (ab39362; Abcam, Cambridge, MA) at a 1:100 dilution and a rabbit polyclonal antibody to thromboxane A₂ receptor (MBL International Corporation, Woburn, MA, LS-A1278) was used at a 1:250 dilution. A pre-immunization rabbit polyclonal antibody (Abcam, Cambridge, MA, ab27478) was used as an isotype control for both the thromboxane synthase and thromboxane A₂ receptor at a 1:100 dilution. The secondary antibody was a goat anti-rabbit IgG-Fc Fragment – FITC (Jackson Immuno Research Labs, West Grove, PA) at a 1:100 dilution.

2.3. Immunofluorescence Image Analysis

Images were analyzed using NIS Elements BR 3.0 software (Nikon Instruments Inc., Melville, NY). Three sections per central region and three sections from the peripheral region were captured for analysis. Mean intensities from the three central and three peripheral regions were averaged for each layer of the retina and for each animal. The analysis for the central and peripheral retina was broken into five regions of interest (layers): the ganglion cell layer + inner plexiform layer (GCL + IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and photoreceptor – inner segment (PR-IS). The retinal pigment epithelium (RPE) becomes detached during processing and was not always present on the slides; therefore, this layer was only qualitatively analyzed. Computer image analysis of each layer was completed using the following steps: 1) the DAPI-counterstain images were enlarged, 2) regions of interest were drawn around the INL, OPL, and ONL, 3) these regions of interest were pasted into the FITC-stained images, with fluorescence intensity measured for each, 4)

the final two regions of interest (GCL+IPL and PR-IS layers) were drawn in the FITC image, with intensities measured, and 5) a background region of interest was analyzed for each image captured. This procedure was completed for the central and peripheral retina. Corrected mean intensity was calculated as follows: (mean intensity from region of interest – background intensity for that image) / exposure time / mean light intensity.

2.4. Statistics

Results are expressed as mean $\pm$ standard error of the mean. Statistical analyses (T-tests) were performed with GraphPad Instat version 3.05 software (San Diego, CA). A p-value < 0.05 was considered statistically significant. When standard deviations were considered significantly different, a Welch correction was performed on that T-test. An ANOVA with Bonferroni correction was used when multiple groups were being compared. Due to a few poor quality tissue sections from diabetic rats, two data points were eliminated from the thromboxane synthase analysis, and one data point was eliminated from the thromboxane receptor analysis.

Results

3.1. Animal Data

Data on body weight and glucose values for each group of mice and rats are presented in Table 1. Included in the study were 8 non-diabetic control mice, 7 STZ-diabetic mice, 8 non-diabetic control rats, and 12 STZ-diabetic rats. A 3–5 fold increase in glucose levels were induced in STZ-injected mice and rats compared with buffer-injected controls. By day 6 post-injection, no STZ mice had glucose values > 600 mg/dl (the upper limit of the glucometer); however, by the end of the fourth week 3/7 mice had values exceeding this value. Day 6 post-injection, 1/10 rats had glucose values > 600 mg/dl, with this fraction increasing to 4/12 by the end of the fourth week (data were not obtained from two rats on day 6). Due to the values > 600 mg/dl (specific value unknown), we present median levels of blood glucose in Table 1.

As expected, STZ-injected mice had a significant decrease in weight compared to the day of injection (Table 1). Unlike STZ mice, STZ rats continued to gain weight compared to the day of injection, although not as large of an increase in weight as in buffer-injected rats.

3.2. Thromboxane Synthase

Figure 1 provides the fluorescent staining intensities for thromboxane synthase in the retinas of mice (Fig 1A) and rats (Fig 1B). The higher staining intensities were in the ganglion cell + inner plexiform layers and in the outer plexiform layer, with more staining in the inner nuclear layer than in the outer nuclear layer. Two types of negative controls (no primary antibody and a non-immune isotype antibody) had insignificant levels of staining (data not shown). In both mice and rats, the lowest staining for TxS was found in the two outer layers, that is, the outer nuclear layer and the photoreceptor inner segment. As shown in Figure 1, four weeks of STZ-induced diabetes did not alter the levels of retinal TxS. Representative images of TxS staining (from control animals) are shown in Figure 2. Of interest, TxS staining was evident in the retinal pigment epithelial layer of the rat (not quantified), but not in the mouse.

3.3. Thromboxane Receptor

Figure 3 provides the fluorescent staining intensity values for the TP receptor in the retinas of mice (Fig 3A) and rats (Fig 3B). In contrast to thromboxane synthase, the lower staining intensities were observed toward the inner retina (ganglion cell + inner plexiform layers and inner nuclear layer), with the highest staining intensity in the photoreceptor inner segment. Two types of negative controls (no primary antibody and a non-immune isotype antibody) had insignificant levels of staining (data not shown). The intensities of staining in mice appeared

to be virtually identical between controls and diabetics, and the general pattern of staining in rats was also similar between the two groups. However, in diabetic rats, variability in the TP receptor gradually increased toward the outer retina, with significantly higher (3–4 fold) standard deviations in the intensity levels (Fig 3C). In fact, from the photoreceptor inner segment, the staining intensities of the diabetic rats fell completely out of the range of values found in controls (54–70 intensity units), with 8/11 data points within a range of 72–103 and the remaining 3 data points in the range of 27–52. Representative images of TP receptor staining (from control animals) are shown in Figure 4. As with TxS, the TP receptor stained positively in the retinal pigment epithelial layer of the rat, but not in the mouse.

Discussion

To our knowledge, this is the first report of the localization of thromboxane synthase and receptor in the retina of mice or rats. Immunostaining for both the synthase and receptor were found in all retinal layers of both species, with the staining patterns very similar between mice and rats. The most intense staining for the synthase was found in the ganglion cell/inner plexiform layer and in the outer plexiform layer, and the most intense staining for the receptor was found near the edge of the outer retina in the photoreceptor inner segment.

Two differences between mice and rats were observed in the immunohistochemical analyses. Firstly, positive staining for thromboxane synthase and its receptor were observed in the retinal pigment epithelial layer of rats, but not of mice. Secondly, induction of STZ-induced diabetes did not alter expression of the synthase or receptor in mice, but did alter the expression of the receptor in rats. The nature of the alteration in rats was that of a substantial (3–4 fold) increase in variability in the outer retina; however, the causes of the increased variability remain unknown. With the variability, most of the diabetic rats demonstrated an increase in TP receptor staining, possibly consistent with a role for thromboxane in cellular dysfunction induced by hyperglycemia.

Most reports of a pathological role for thromboxane in the retina (whether in diabetes or in other conditions) focus on the microvasculature. As described in a comprehensive review (Hardy et al., 2005), thromboxane can induce vasoobliteration; additionally, thromboxane induces time- and concentration-dependent retinovascular cell death, primarily by necrosis (rather than apoptosis). However, nerve cells also have been shown to produce and have receptors for thromboxane (Giulian et al., 1996; Honma et al., 2006), and binding of the TP receptor induces major morphological changes in human astrocytoma cells (Honma et al., 2006).

Our findings of intense staining of the TP receptor in the photoreceptor layer in mice and rats is consistent with TP receptor staining reported in the photoreceptors of the human retina (Chen et al., 1994). Other than our current study, any additional information on the retinal localization of thromboxane synthase and its receptor appears to be scarce. However, reports from the literature describe across many species (cats, cows, humans, rabbits, rats) the ability for the retina to produce thromboxane (Birkle and Bazan, N. G., 1984; Kulkarni, 1991; Preud'homme et al., 1985; Setty et al., 1991). In incubated rat retinas (Birkle and Bazan, N. G., 1984), concentrations of TxB₂ were higher than any other prostanoid, with 6-keto-F1 α (the downstream product of prostacyclin) a close second, and with no other prostaglandin in the same range of concentrations. Whether the bulk of this retinal thromboxane is derived from platelets or from neural cells is undetermined; however, our staining patterns for thromboxane synthase in the mouse and rat retina suggest that neural tissue could be a major source.

It was interesting to note that the inner retina had relatively high thromboxane synthase expression but low TP receptor expression; and in contrast, the photoreceptor inner segment

had the highest TP receptor expression but relatively low synthase expression. Although highly speculative, it could be hypothesized that this inverse relationship maintains fairly similar levels of thromboxane signaling, that is, there could be less need for high expression of the TP receptor in areas of high synthase expression. The one notable exception to this inverse relationship was the outer nuclear layer, which had relatively low staining of both the synthase and receptor.

Future work is needed to determine the cellular sources and time course of potential changes in TxS and TP receptors due to hyperglycemia. Based on our previous findings that retinal arteriolar diameters are decreased early in the diabetic retina and that this vasoconstriction can be attenuated with a thromboxane synthase inhibitor or TP receptor antagonist (Lee et al., 2008; Lee and Harris, N. R., 2008; Wright et al., 2009; Wright and Harris, N. R., 2008), it is possible that early changes in thromboxane synthase and/or TP receptor density may occur in vascular cells before any potential changes occur in the neural retina.

In summary, we have shown that thromboxane synthase and the TP receptor are present in all layers of the mouse and rat retina, with an interesting inverse relationship between the synthase and receptor in most layers. Streptozotocin-induced diabetes had very little influence on the immunostaining patterns, with the exception of a high variability for the TP receptor in the photoreceptor inner segment of rats.

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Figure 1.

Retinal thromboxane synthase fluorescent staining from frozen cross-sections of control and diabetic mice and rats. The black line represents the mean value for each data set. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, and PR-IS = photoreceptor inner segment.

Figure 2.

Representative cross-sectional images of retinal thromboxane synthase staining (green; FITC) in the mouse (panel A) and rat (panel D). Panels B and E show the DAPI counter-staining for A and D, respectively. Panels C and F are corresponding FITC / DAPI merges. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, PR-IS = photoreceptor inner segment, and RPE = retinal pigment epithelium.

Figure 3.

Retinal TP receptor fluorescent staining from frozen cross-sections of control and diabetic mice and rats (panels A & B, respectively). The black line represents the mean value for each data set. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, and PR-IS = photoreceptor inner segment. Panel C gives the standard deviations of the data from panel B. ** $p < 0.01$ and *** $p < 0.001$ vs the corresponding control.

Figure 4. Representative cross-sectional images of retinal TP receptor staining (green; FITC), along with the corresponding DAPI counterstaining (blue) and merged FITC/DAPI images.

Body weight and blood glucose values in the experimental groups: control mice (N=8), streptozotocin-injected mice (STZ; N=7), and STZ-injected rats (N=12). Glucose levels are reported as median values.

Table 1

	Group	Day of injection		Day 6 after injection		Week 4 after injection	
		Control	STZ	Control	STZ	Control	STZ
Weight (g)	4 week – Mice	26.7 ± 0.4	26.2 ± 0.5			25.2 ± 0.6	18.7 ± 0.4***
	4 week – Rats	147.5 ± 4.2	147.8 ± 3.2			342.8 ± 11.0 ^{†††}	231.7 ± 7.6***
Glucose (mg/dl)	4 week – Mice			174	541	142	539
	4 week – Rats			126	431	153	564

*** p<0.001 vs respective day of Control, and p<0.001 vs STZ day of injection.

^{†††} p<0.001 vs control day of injection.