EXAMINATION OF THE RELATIONSHIP BETWEEN GLUCURONIC ACID AND VASCULAR DAMAGE IN RATS

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The goal of this experiment was to examine the role of glucuronic acid in the development of vascular damage in the kidneys and retinas of diabetic individuals. Glucuronic acid was provided to rats in their water at various concentrations in order to increase plasma levels of the compound. Kidneys and retinas were excised and compared to control specimens using microscopy to determine the effect of elevated blood glucuronic acid levels on the occurrence of microaneurysms in renal capillary networks. No differences were seen between the treatment and control groups. Further study needs to be conducted to determine a more suitable time frame for this experiment.
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INTRODUCTION

Diabetes mellitus is one of the leading causes of death around the world. This disease accounts for approximately 70,000 deaths each year (Danaei et al. 2009) and direct and indirect economic costs of diabetes in 2002 were estimated to be 132 billion dollars in the United States alone (Hogan et al. 2003). The prevalence of this disease is increasing rapidly throughout the world and both the economic cost and mortality rates continue to rise.

Two types of diabetes exist: type I diabetes and type II diabetes. Type I diabetes begins when β-cells in the pancreas are destroyed autoimmunologically. β-cells produce insulin and their loss results in lowered insulin levels and elevated blood glucose concentrations. Type II diabetes results when the sensitivity of cells to insulin is diminished, tissues take up less glucose, and blood glucose concentration increases. The World Health Organization estimates that diabetes type II accounts for 90% of all cases of diabetes mellitus (World Health Organization 2012). Type II is life style related and its incidence rises with obesity rates. The major complications of both types of diabetes are the result of microvascular damage which leads to tissue hypoxia, such as occurs in diabetic retinopathy, neuropathy and nephropathy or alterations in the macrovasculature which affect blood flow to the heart, brain, and lower extremities leading to an increased risk of limb amputation, stroke, and myocardial infarction (Brownlee 2001).

Diabetic vascular damage begins early during the course of the disease. Thickening of the basement membrane occurs resulting in increased vascular permeability (Roy et al. 2010). Increased vascular permeability also occurs due to diminished vasodilator activity and augmented vasoconstrictor and permeability factor activity (Brownlee 2001; Lopez-Quintero et al. 2010). Hematologic abnormalities such as reduced red blood cell deformability and
aggregation of erythrocytes and platelets occur that reduce blood flow. Vascular permeability is further affected by the selective loss of pericytes. Microaneurysms form in the capillary network often followed by thrombus formation and vessel occlusion. This leads to hypoxia, tissue damage, and neovascularization (Jie et al. 2010). Atherosclerosis associated with diabetes starts with endothelial dysfunction. Vascular endothelial cells produce less nitric oxide, which is an anti-atherogenic compound. Smooth muscle cell proliferation and potentiation increases (Jie et al. 2010) which causes higher than normal sympathetic tone.

There are currently four main hypotheses of the biochemical origins of this damage: increased polyol pathway flux, increased advanced glycation end-product (AGE) formation, activation of protein kinase C isoforms, and increased hexosamine pathway flux (Brownlee 2001). The polyol pathway is responsible for the enzymatic conversion of glucose into sorbitol by aldose reductase. Hyperglycemia results in an increase of this reaction due to elevated substrate concentration. Detrimental effects of increased flux of this pathway are not well understood and seem to vary depending on species and tissue tested (Brownlee 2001). When aldose reductase was blocked in diabetic dogs, neuropathy was prevented but not the development of microvasculature damage (Engerman et al. 1994). Another study with diabetic mice blocked aldose reductase to reduce polyol and reduced mesangial expansion (Hashimoto et al. 2011). The second hypothesis deals with the AGE formation. Concentrations of AGEs are higher in retinal vessels and glomeruli of diabetics and there is evidence that AGEs are involved in diabetic microvascular disease: the development of diabetic glomerulosclerosis in rats was retarded using AGE inhibitors (Nakamura et al. 1997). AGE inhibition has also been shown to slow the progression of diabetic nephropathy and retinopathy in human patients (Brownlee 2001). AGEs pathologically modify gene expression and modify proteins altering their function.
Furthermore, AGE precursors modify extracellular matrix components and alter the way they interact with matrix proteins (Brownlee 2001). A recent study linked the restriction of AGE’s to an improvement in insulin resistance in diabetes type II patients (Uribarri et al. 2011). Protein kinase c is the focus of the third main hypothesis. Altered protein kinase c (PKC) activation has been associated with decreased nitric oxide (NO) production in smooth muscle and glomeruli. PKC-β inhibitors have been effective in improving glomelular filtration and retinal circulation in diabetic animals (Brownlee 2001). The final main hypothesis deals with the hexosamine pathway. Hyperglycemia leads to an increase in hexosamine pathway activity and results in changes in gene expression and protein function that may result in the development of diabetic pathologies (Brownlee 2001).

Glucuronic acid is a metabolite of glucose that is produced in a process known as the glucuronic acid cycle. This pathway occurs in several places in the body including the intestine and hepatocytes and all steps in this cycle have been shown to take place in arterial tissue (Ritz and Sanwald 1970). Plasma glucuronic acid concentration is higher in diabetic individuals (Chorne Navia et al. 1999) and this elevation has been attributed to an increase in the activity of UDPGA-pyrophosphatase which forms D-glucuronic acid through hydrolysis of UGDPA, and D-glucuronic acid-1-phosphate phosphatase which dephosphorilates D-glucuronic acid-1-phosphate and a defect in L-gulonic acid dehydrogenation (Hinohara et al. 1974). It is currently unknown if or how glucuronic acid is associated with the current hypotheses outlined above; however, the flux of both the polyol and glucuronic acid pathways increase with glucose concentrations and cause flow and pressure changes by influencing the endothelium (La Fontaine et al. 2006). Additionally, the hexosamine pathway is involved in the formation of proteoglycans and may effect the formation of proteoglycans formed from glucuronic acid.
Glycosaminoglycans, such as hyaluronic acid, chondroitin sulfates, heparin, and heparan sulfate are important for various aspects of cardiovascular physiology. These polysaccharides contain varying amounts of glucuronic acid. Chondroitin sulfates are a vascular extracellular component important for the structural integrity of blood vessels. Chondroitin sulfate is the principal component of basement membrane specific-chondroitin sulfate proteoglycan, which has been identified in most basement membranes with the exception of non-diabetic glomerular capillary basement membranes (McCarthy et al. 1994). Basement membrane-specific chondroitin sulfate proteoglycan has been observed in the glomerular capillary basement membranes of diabetic rats (McCarthy et al. 1994). Heparin binds to angiogenic growth factors and stimulates growth of new blood vessels. Both heparin and hyaluronic acid are vascular extracellular matrix components and have been shown to regulate vessel growth and proliferation by inhibiting the attachment of pericytes and smooth muscle cells while enhancing capillary endothelial cell attachment (Orlidge and D'Amore 1986). Heparan sulfate occurs in perlecan, a proteoglycan of the extracellular matrix that inhibits smooth muscle cell proliferation (Brownlee 2001). The hyperglycemia induced reduction in expression of perlecan on hepatocytes leads to an increase in cholesterol-enriched apolipoprotein B-containing remnant particles. High levels of these particles are a risk factor for atherosclerosis (Ebara et al. 2000). Elevated glucose levels have been linked to alterations in the glycosaminoglycan composition of the human glomerular endothelial glycocalyx with a specific reduction of heparin sulfate (Singh et al. 2011).

Previous studies have shown that diabetic patients exhibit as much as 100% higher plasma glucuronic acid concentrations than non-diabetics (Chorne Navia et al. 1999). It has also been shown that rats developed retinal microaneurysms similar to what would be seen in diabetic retinopathy when given high concentrations of glucuronic acid in their water, which would be
expected to raise glucuronic acid plasma concentrations (Chorne Navia, Cisneros et al. 1997). The development of glomelular microaneurysms has also been observed in kidneys of diabetic animals (Nakagawa et al. 2007). Furthermore, patients suffering from diabetic retinopathy had improved vision after being treated with the drug piperazine which is known to reduce plasma glucuronic acid levels (Chorne Navia et al. n.d.).

The glycosminoglycan content of retinal basement membranes differ from those of the glomerulus. There are also differences in the amount and function of pericyte cells in these two tissues. Due to differing composition of basement membranes based on tissue type and the fact that glucuronic acid may interact with basement membrane compounds to initiate vascular lesions, differences in the progression of damage may be observable in the two tissues.

The goal of this study was to corroborate and expand upon the findings of Chorné et al. (1997) to provide a platform from which to conduct further study into the underlying mechanisms of the interactions that associate glucuronic acid with vascular damage. This previous study of glucuronic acid and diabetic microvascular disease dealt mainly with microaneurysms occurring in the retina; however, qualitative observations of changes in the capillary network of the kidney were also discussed (Chorne Navia et al. 1997). A goal of the current study was to further quantify and characterize these observations, which will lead to a better understanding of the mechanism behind diabetic microaneurysms, and could ultimately yield better treatments and diagnostic methods for diabetes mellitus. Therefore, the hypotheses for this study were:

Hypothesis I: The development of microvascular damage in the kidney and retina will increase when concentrations of glucuronic acid are provided in the drinking water of rats.
Hypothesis II: Vascular damage associated with glucuronic acid is reversible and will diminish upon removal of glucuronic acid from the water.
METHODOLOGY

Animal Treatments

Female Sprague Dawley rats (Rattus norvegicus) were used as the model organism for this study. A control group of 7 rats was provided normal water, while 3 test groups of 7 animals were provided unlimited access to water containing glucuronic acid at concentrations of 120, 80, and 40mg per 50ml water. After 4 weeks, animals were sacrificed and the kidneys and retinas were removed and examined microscopically for the presence of microvascular lesions. All animals were weighed on a weekly basis throughout the study.

To determine if this damage was reversible, a second control group was provided normal water, while 2 test groups of 7 animals were provided glucuronic acid at concentrations of 80, and 40mg per 50ml of water daily. After 4 weeks, glucuronic acid was removed from their water and the animals were provided normal water for 4 more weeks. After the 8 weeks the animals were sacrificed, and the kidneys and retinas were removed and examined microscopically for the presence of microvascular lesions.

Renal Histology

After fixation in a 4% paraformaldehyde solution, the kidneys were dehydrated with Prowave solution and embedded in paraffin using an RHS microwave histoprocessor. Fixed kidneys were sliced into 5µm sections, stained with periodic acid-Schiff reagent, and counterstained with hematoxylin (Sigma-Aldrich). The preparations were then inspected for evidence of glomerular sclerosis, specifically, focal segmental hyalinosis and the presence of microaneurysms. Basement membrane width was measured with ImageJ software. Basement
membranes that were cut obliquely or appeared distorted due to slicing were excluded. Three slides were examined for each animal with the average of 4 measurements taken from the basement membrane surrounding a capillary loop. Four capillary loops were examined per slide.

Retinal Histology

Whole eyes were fixed in 4% paraformaldehyde solution. Retinas were separated and post-fixed in methanol at -20°C and then rehydrated in PBS Triton x100 1% pH 7.3 solution and incubated overnight in a 1:100 solution of FITC conjugated lectin from *Banderraea simplicifolia* (Kowalczuk, et al. 2011). Mounted slides were examined with a flourescence microscope at a wavelength of 395nm for the presence of microaneurysm like structures in the microvasculature. Microaneurysm like stuctures were counted per capilary branch and 4 capilary branches were counted per retina.

Statistical Analysis

The average number of microaneurysm per retina for each group were compared with an ANOVA. Statistical signifiance was assumed at $P < 0.05$. Data is reported as mean ± standard deviation.
RESULTS

Body mass

The mean body mass for each group are shown in Table 1. The control group started out smaller than the treatment groups and remained so throughout the experiment. At week 1, the control group was significantly lighter than the 40 group ($P=0.018$). There were no significant differences between the groups at week 4 ($P=0.058$). Both the 40 group and the 80 group were significantly larger than the control at week 8 ($P=0.003$).

Kidneys

As seen in Figure 1, no differences were observed in the glomerular morphology between the control and treatment groups after 4 weeks of treatment. The arrows show the mesengial matrices of the control glomeruli are of normal size, podocytes are present and the capillaries are thin. The same is true for each treatment group. The presence of microaneurysm like structures, as well as focal segmental hyalinosis, were not observed in the kidneys of any group. All groups have what appear to be healthy glomeruli. There were no significant differences in capillary basement membrane width between the groups (Figure 2; $P=0.196$).

Retinas

As seen in Figure 3, the retinas were likewise free of microaneurysms. The control and treatment retinas appear healthy, with no visible abnormalities. If present, microaneurysms appear as balloon-like projections from the capillaries and are easily visualized with this technique,
however, the vessels from this experiment appear smooth, with no apparent bulges or projections. There were no significant differences in the number of microaneurysm like structures per retina between the groups (Table 2; \( P=0.261 \))

Table 1. This table displays the mean mass (g) ± SD of the control, low treatment (40mg GA/50ml H\(_2\)O), medium treatment (80mg GA/50ml H\(_2\)O) and high treatment (120mg GA/50ml H\(_2\)O) groups at weeks 1, 4 and 8. *P=0.008, ** P=0.007, ***P=0.003

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>t40</th>
<th>t80</th>
<th>t120</th>
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</thead>
<tbody>
<tr>
<td>Week 1: Mass (g)</td>
<td>254.2± 10.2</td>
<td>257.7± 7.5*</td>
<td>263.4± 11.7</td>
<td>268.6± 14.4</td>
</tr>
<tr>
<td>Week 4: Mass (g)</td>
<td>328± 18.6</td>
<td>346.2± 25.9</td>
<td>334.1± 19.7</td>
<td>322.9± 15.6</td>
</tr>
<tr>
<td>Week 8: Mass (g)</td>
<td>374.9± 15.9</td>
<td>401.1± 10.9**</td>
<td>407.6± 21***</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Week 4 Glomeruli. (A) Control (B) 120mg GA/ 50ml H₂O (C) 80mg GA/ 50ml H₂O (D) 40mg GA/ 50ml H₂O. There is no observable focal segmental hyalinosis or microaneurysms in any. Arrows show (1) Messengial matrix cells (2) Podocyte (3) Capillary loop
Figure 2. Mean glomerular basement membrane width for each group. Control 0.58 ± 0.06, Low (t 40) 0.61 ± 0.03, Medium (t 80) 0.57 ± 0.04, High (t 120) 0.56 ± 0.03
Figure 3. Week 4 Retinas. (A) Control (B) 120mg GA/ 50ml H₂O (C) 80mg GA/ 50ml H₂O (D) 40mg GA/ 50ml H₂O. There are no microaneurysm like structures visible in any.

Table 2. Mean number of microaneurysm like structures per retina of the control, low treatment (40), medium treatment (80) and high treatment (120) groups at weeks 4 and 8.

<table>
<thead>
<tr>
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<th>Control</th>
<th>t 40</th>
<th>t 80</th>
<th>t 120</th>
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</thead>
<tbody>
<tr>
<td>Number of Microaneurysms at week 4</td>
<td>0.083</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
DISCUSSION

The absence of damage could be explained by the duration of the experiment: the time frame may have been too short for observable damage to occur. Nearly all of the rats in the previous study were completely blind after 60 days of treatment (Chorne Navia et al. 1997). The time frame of the current experiment was reduced in order to better assess the reversibility of the damage. This is because tissue damage allowed to progress to such an extent as that of previous experimental animals may not be readily reversible. Enlarged vessels, microaneurysms, edema, and hemorrhages were all observed in the retinas of the experimental animals of the previous study and in the kidneys, hyperplasia of the endothelium of the glomeruli was also observed (Chorne Navia et al. 1997). If a diminished level of damage were achieved, the observation of microaneurysms would be possible as they manifest early during the progression of diabetic retinopathy and nephropathy. Another possible explanation of the absence of damage is that plasma glucuronic acid levels were not elevated sufficiently by the delivery method. These levels will need to be determined by LSMS in order to exclude this possibility. It is unknown how much of the glucuronic acid consumed is absorbed into the blood stream or how much is excreted through first pass metabolism. Pharmacokinetic examinations should be carried out to determine the half-life of glucuronic acid in this model system. This will make correlation of vascular damage with specific plasma concentration possible. Delivery via an I.V. drip may be more efficacious in achieving a stable and precise plasma concentration. Furthermore, it was impossible to precisely determine how much solution each individual rat consumed, because 2-3 rats shared a water bottle and each bottle leaked slightly. The use of an I.V. would eliminate these issues. This method may present its own problems such as increased cost and removal of the I.V. by the rats. It may also be possible to mix a precise dosage into a “treat” such as a small
amount of peanut butter. Rats could be observed consuming each dose in their entirety. This however, would likely need to be carried out daily or even multiple times each day, making this method more impractical.

If the delivery method is determined to be effective, then the current study should be repeated using a longer time frame. Also, the 120mg group should be excluded or replaced with a group given a lower concentration. This group drank less of its solution, thereby consuming approximately the same amount of glucuronic acid as the 80mg group. Treating 3 groups with concentrations of 40, 60, and 80mg glucuronic acid/50ml H₂O for a time period of 6-8 weeks should increase the likelihood of observing reversible damage.

Further study is needed to determine the pathway by which glucuronic acid influences the development of microvascular damage. Determination of the affect of elevated plasma glucuronic acid on the formation of glycosaminoglycans is necessary for better understanding of the progression of this disease. This is especially important considering the role that these compounds have on structural integrity of vessel walls. Hyaluronic acid may be particularly important not only because of its role in vascular extracellular matrix, but also due to its accumulation during the development of diabetic microangiopathy. Furthermore, researchers have postulated that thickening of the basement membrane is due to either a decreased rate of the removal of basement membrane material or an increase in its production (Lazarow and Speidel 1964). More research must be conducted to better understand if increased plasma glucuronic acid concentrations lead to an increase in hexosamine pathway activity and polyol pathway flux and if these in turn affect development of the capillary basement membrane. The majority of work done on diabetic microangiopathy focuses on the affect of increased glucose concentration without considering the role of glucuronic acid; therefore, more research is needed to determine if
elevated glucuronic acid has a separate effect. Increased glucose concentration has been shown to alter the composition of glycosaminoglycans in the glomerular endothelial glycocalyx (Singh et al. 2011). Work needs to be done to determined if elevated glucuronic acid has a similar effect and if these alterations to the glycocalyx can lead to microvascular lesions. It is also important to consider the role glucuronic acid plays in diabetic atherosclerosis. This could be accomplished by raising glucuronic acid levels, then testing vascular reactivity using a force transducer.

Alterations to the hexosamine pathway have been shown to decrease vascular reactivity by impairing dialation via nitric oxide (Beleznai et al. 2012). If glucuronic acid concentration affects the hexosamine pathway then it would be involved in this aspect of diabetes pathology as well.

While the expected results were not achieved by this study, it was successful in laying the foundation for future experimentation on this topic. To begin testing the pathways mentioned earlier, a functional experimental framework in which observable and reversible microvascular damage can be elicited will be indispensable. The work described here has made progress toward developing this framework. This study has the potential to introduce a novel approach to diabetes research and provide the foundation for the development of better treatment for this impactful and debilitating disease.
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