Does Nitric Oxide (NO) and/or Superoxide (.O$_2$-) Cause Type 2 Diabetes and can it be Prevented?

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Abstract

Diabetes 2 is caused by metabolic effects on the pancreatic ß cells with loss of insulin sensitivity or effectiveness and control of its secretion. Some damage to the Beta cells probably occurs continuously once the disease process starts and oxidative and nitrosative stresses likely play a role as well caused by the proper lack of glucose control. Recent work indicates that excessive glucagon from alpha cells which replace Beta cells have an important role raising glucose levels. If this scenario is correct, specific antioxidant substances might have an important potential to quench, degrade or react with the key substances causing oxidative stress via oxidation/nitration processes causing chemically induced diabetes namely, superoxide (.O$_2$)–, nitric oxide (NO·), peroxynitrite (OONO-), etc. This pre-exposure may be able to prevent type 2 diabetes mellitus and/or its clinical consequences.

Previously we found that correct doses of carboxy-PTIO (sodium salt) prevented diabetes mellitus I caused by Streptozotocin (STZ) in rats. Carboxy -PTIO oxidizes excessive nitric oxide from STZ occurring in the Beta cell. An intermediate dose of (STZ) in rats should partially mimic a type 2 diabetic state (glucose levels approx 300 mg/dl vs. 100 mg/dl in normal animals. We added effective antioxidants tempol and acetaminophen to prevent any excessive oxidative damage from STZ.

Introduction

Regardless of the type of diabetes, decreased insulin and excessive blood glucose levels eventually contribute to vascular damage as a central basis to the pre-pathogenesis of the many pathological consequences of diabetes mellitus. In type I diabetes, the death of pancreatic βcells prevents insulin production, storage and release which causes vascular damage and toxicity to organs and diabetic damage results. If β cell death could be prevented or delayed, type I diabetes should be prevented or at the least ameliorated. Type I diabetes mellitus appears to occur when greater than 80% of the pancreatic βcells are destroyed [1]. It appears as if nitric oxide (NO.) and superoxide, (.O$_2$) -or peroxynitrite produced within the βcell (chemical hypothesis) or externally by macrophages activated by cytotoxic T cells (the immune mediated hypothesis) all appear to be immediate agents responsible for pancreatic β cell injury and death [2]. Similar chemical processes appear to happen in type 2 diabetes but generally at a slower rate. Beta cells can die almost completely in type 2 diabetes and eventually these patients must receive large doses of insulin to control blood sugar levels.

Pancreatic β cells are rich in mitochondria, a rich intracellular source of superoxide production [3,4]. Mitochondria also produce nitric oxide and peroxynitrite resulting from the reaction of nitric oxide with superoxide which is likely to be generated within pancreatic β cell mitochondria. Peroxynitrite, however, is very much more damaging to cells, causing nitration, oxidation and PARP activation, indirect DNA damage and apoptosis or cell death [5-9]. Other combinations of oxygen and nitrogen may be involved in toxicity as well as peroxynitrite eg., Nitrogen dioxide, etc.

Not only are pancreatic βcells able to produce toxic free radicals or their toxic reaction combination products such as NO., superoxide and combination products eg. peroxynitrite etc., rats have a limited supply of endogenous enzymatic and antioxidant mechanisms [4] making them highly susceptible to oxidative and nitrosative stress and subsequent Beta cell injury or death. Excessive nitric oxide or a combination of nitric oxide and superoxide creates peroxynitrite (OONO-), or NO$_2$ and N$_2$O$_4$ [10-12]. In chemically-induced type I diabetes caused by

streptozotocin, multiple investigators using various substances which interfere with nitric oxide have partially prevented [13-19] or mostly prevented type I diabetes mellitus. Streptozotocin has been shown to generate nitrite and nitrate inside β cells [20]. Mice that are NO synthase II knock out animals are not very effective at developing diabetes [21]. In contrast; mice genetically engineered to produce excessive nitric oxide develop diabetes without streptozotocin [22]. All of these data suggest that excessive nitric oxide directly or indirectly causes β cell death in both types I and type 2 diabetes mellitus.

Regardless of whether the cause of the diabetes is genetic, environmental or chemical, nitric oxide is apparently a key factor. Furthermore, superoxide appears to be involved as well. If these two chemicals have an actual role in the pathogenesis of type I and type 2 diabetes mellitus, drugs which chemically eliminate or destroy (NO.) and (.O$_2$) - or act on scavenger targets for oxidation and nitration might prevent or ameliorate both types of diabetes mellitus.

It is well known that sodium carboxy-PTIO can react with nitric oxide (NO.) producing (.NO$_2$) and carboxy-PTI. This reaction is shown below:
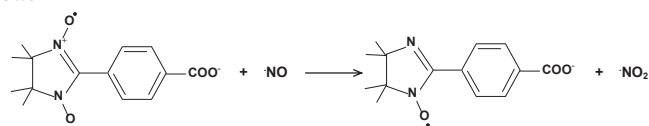
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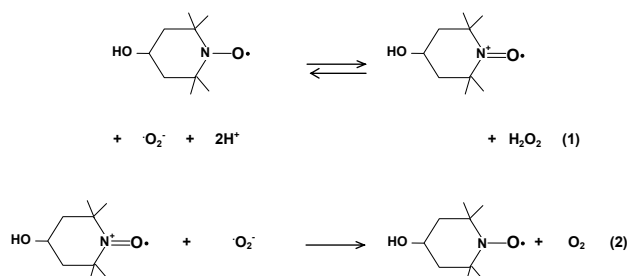
This reaction effectively prevents nitric- oxide- induced cytotoxicity by oxidizing nitric oxide to nitrite radical.4-OH tempol reacts with superoxide producing hydrogen peroxide and recycling back to 4-OH tempol.

This reaction is shown as follows:

The products of 4-hydroxy tempol reactions (1) and (2) react as follows:

$$2H^+ \;+\; 2\,.O_2^- \;\longrightarrow\; H_2O_2 \;+\; O_2$$
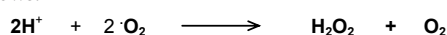
Acetaminophen (Tylenol™) can act as an antioxidant and an anti-nitrating substance by acting as a target for peroxynitrite which inhibits the oxidative and nitrosative stress experienced by a cell [14]. In addition, it inhibits the nitration of proteins containing tyrosines or tryptophanes further reducing the oxidative and nitrosative stress experienced by a cell.

Szabo C, Kiss et al. [6,7] have shown that peroxynitrite (OONO-) has a pathogenic role in the development of diabetes and its vascular complications with the use of a peroxynitrite scavenger, and specifically they were able to inhibit tyrosine nitration and peroxynitrite induced cytotoxicity *in vivo* in type I diabetes mellitus and STZ treated rats. In these experiments, FP15 treatment (3-10 mg/kg), a novel potent peroxynitrite decomposition catalyst, reduced the incidence of diabetes mellitus in rats subjected to multiple low doses of streptozotocin. Moreover, they reported a potential reduction in the vascular and cardiac effect of diabetes mellitus in mice [6].

A combination of antioxidant and anti-nitrating substances in rats might be able to prevent streptozotocin induced diabetes 2 and its complications. The purpose of this paper is to investigate this proposition and evaluate potential mechanisms causing a form of diabetes 2 which might lead to corrective substances useful in man.

Methods

Drugs and chemicals

Carboxy-PTIO (sodium salt) was obtained from the Cayman Chemical Company, Ann Arbor, MI.4-OH tempol and streptozotocin, HEPES and Phosphate Buffered Saline (PBS) were purchased from Sigma/Aldrich Chemical Company, St. Louis, MO. Acetaminophen (Tylenol®) was obtained from the McNeil Company, Fort Washington, PA.L-012 was purchased from Waco Chemical Company, Virginia. SIN-1 was obtained from Dr. Karl Schönafinger, Casella AG, Frankfurt, Germany. The chemical structures of carboxy-PTIO, 4-OH tempol and acetaminophen and streptozotocin are shown in Figure 1.

Drug treatment of animals and glucose monitoring

The animals used in the following experiments were housed and treated according to the rules of the West Virginia University animal care committee. Sprague-Dawley male albino rats weighing about
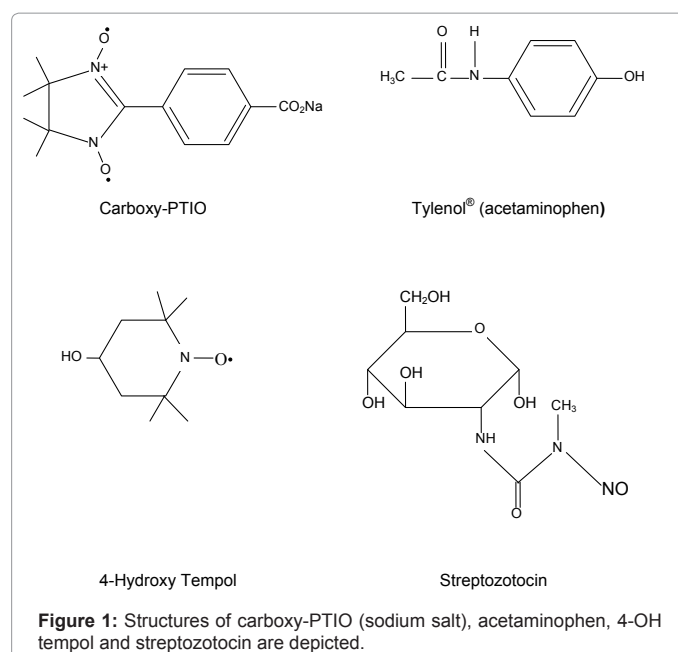
Figure 1: Structures of carboxy-PTIO (sodium salt), acetaminophen, 4-OH tempol and streptozotocin are depicted.

300 grams were used. Six rats were injected subcutaneously with a combination of streptozotocin at 30 mg/kg and 10 mg acetaminophen, 10 mg carboxy-PTIO and 10 mg 4-OH tempol dissolved in 0.1 M Phosphate Buffered Saline (PBS).Six control rats were injected with 30 mg/kg streptozotocin in (PBS) alone and 6 animals were treated with STZ and carboxy-PTIO as drug treated control. The data from the drug treated control was almost identical to the combination therapy (data not shown). This clearly indicates the key ant diabetic ingredient was carboxy-PTIO. The animals were examined 24 hours and 3, 6, and 12 months later and had their blood analyzed for glucose via the tail vein using a Thera Sense Flash glucose monitoring system identical to the glucose testing strips used to measure blood glucose in humans.

Specifically blood was obtained from the end of the barely snipped tail of a rat after first applying a local anesthetic salve used in clinical dentistry for surface anesthesia of the gingiva. The Thera Sense glucose monitoring system is effective using a very small drop of blood applied to the edge of a glucose monitoring strip. The meter is calibrated with a known glucose solution prior to assay of blood glucose and after entering the correct numbered test strip number encoded into the device. The test strip number must match the number on the display to obtain as a correct glucose reading.

Control glucose levels in normal and tri-drug protected STZ treated animals

Blood glucose from controls (non-injected animals) or a streptozotocin treated but tri-drug protected animals or drug control animals vary from 70 to 130 mg/dl with the average glucose level being about 100 mg/dl. The glucose level in diabetic animals given streptozotocin alone at 30 mg/kg ranged between 300-400 mg/dl or higher depending on time post injection. The difference in blood glucose levels between STZ alone (diabetic) and non-diabetic STZ + tridrug treated animals was at least four fold. We have also accomplished control studies using STZ- injected animals treated with carboxy-PTIO (same dose as with the combination drug experiments) and glucose stayed normal and none of these animals went blind after

3 months post injections. They also maintained their weight similar to control animals.

LC/mass spectroscopy studies indicate that streptozotocin does not complex with the other drugs. STZ only generates nitric oxide inside the Beta cells because by it electron spin resonance studies indicate that in normal buffered solution there is no generation of nitric oxide. The dose of STZ in the blood of injected animals is the same as the dose of STZ in animals in which the tri-drug combination was used.

Luminescence inhibition measurements to demonstrate antioxidant activity

4-OH tempol, acetaminophen and carboxy-PTIO were assayed for their antioxidant activity using the reaction (Figure 2) between L-012 (a luminol-like compound) and SIN-1 (a generator of peroxynitrite). This combination reacts in a buffered solution to produce an oxidation-based blue luminescence. If an antioxidant is present, either a shift or inhibition of the kinetics of light production will be demonstrated. Luminescence was measured in a Berthold 9505C 6 channel luminometer at 37˚C. The original stock concentration of SIN-1 was 20 mg/5 ml PBS and it was maintained frozen at −80˚C until being used to prevent deterioration. The stock solution was diluted 1:1000 with PBS prior to assay and was maintained in a bucket filled with ice to prevent premature deterioration. It was always added last to the reaction mixture to initiate the reaction. The stock concentration of L-012 is 5 mg/50 mL PBS. The acetaminophen and 4-OH tempol were dissolved initially in a small volume of dimethyl sulfoxide (0.5 ml) and once dissolved was diluted in PBS at various concentrations ranging

Production of peroxynitrite from SIN-1 (linsidomine)

Figure 2: Degradation of SIN-1 (linsidomine) to produce superoxide and nitric oxide free radicals which combine to form peroxynitrite.SIN-1 reacts at physiological pH 7.4 to form peroxynitrite which oxidizes the luminol like compound L-012.

from 1 milimolar to less than micromolar quantities. The volume in each Berthold luminometry tube (3 ml tube) was held constant at 500 microliters in all assays. All drugs and reactants were dissolved in PBS at pH 7.4.The luminescence reaction in each case was maintained for 20 minutes at 37˚C and integrated using the KINB software provided by the Berthold Company, Wild bad, Germany.

Nitration of insulin

Human recombinant insulin (10 mg/ml insulin in 25 mM HEPES pH 8.2) was purchased from Sigma/Aldrich Chemical Company, St. Louis, MO.A buffer with a pH of 8.0 was prepared by titrating with base to 0.1M tris-HCl. A 0.15 mM solution of insulin was prepared by adding 5 mg of insulin to 5 ml of buffer. Tetranitro-Methane (TNM) was used to nitrate the insulin solution.TNM was diluted 1:40 with ethanol.100 µl of TNM were added to 4 ml of pure alcohol. A solution of 3 ml of alcohol and 1 ml of insulin was prepared and 100 µl of alcohol diluted TNM was added to the solution. The TNM-insulin solution was allowed to sit for two hours with excess alcohol being evaporated via a nitrogen blow prior to analysis.

Native gel electrophoresis

Insulin nitration reactions (10 µl) were mixed 1:1 with sample buffer (250 mMTris-CL, pH 6.8, 50% glycerol) and separated on 20% polyacrylamide gels without SDS at 200V for one hour. Gels were stained with Coomassie blue.

Mass spectrometry analysis

Mass spectrometry analysis of the nitrated product was performed on the β-chain of insulin using the proteolytic enzyme trypsin. Gels were stained with Coomassie stain (0.25% Coomassie R-250, 25% isopropanol, and 10% acetic acid) for 20 minutes. Gels were subsequently destained for 1-3 hours in 10% methanol 10% acetic acid (de staining solution changed every 30 minutes). Following destaining, each band was cut and placed into a 0.65 mL low-binding micro centrifuge tube and macerated into ~1 mm² pieces. Gel bands were destained using rotation on an orbital rotator set at 15-20 rpm and then dried. Dried gels were rehydrated and digested with 50 µL of 2 µg/mL (100 ng) trypsin in 25 mM Ammonium Bicarbonate (ABC) overnight at 37˚C. After digestion, the samples were loaded and the resultant peptides were eluted in 4 µl matrix (50 mM α-cyano-cinnamic acid in 49.5% ethanol, 49.5% CAN and 1% TFA). 1 µL of sample and matrix mixture of 3 µl digested peptides were dissolved in 5% acetonitrile with 0.1% acetic and applied to a C18 column using a helium pressure cell applied appropriately for MS/MS assay. After loading, the column was connected to a high pressure liquid chromatography pump with 0.1% acetic acid in channel A and acetonitrile with 0.1% acetic acid in channel B. Peptides were eluted from the column using a linear gradient from 0 to 40% over 40 minutes. The flow rate was set at 250 nL/min, positive spray voltage was 1.2 kV, and the ion transfer tube temperature was 160°C. Masses were measured from 400-2000 m/z and spectra were acquired in a data dependent mode with the instrument performing one full scan followed by three MS/MS scans of the most abundant ions. Raw data were searched against the Swiss Prot database using Sequest states of +1, +2, and +3). Delta CN scores greater than 0.1 were considered statistically significant. A mass shift of 45 Daltons on tyrosine residues was incorporated into the data base search and at least two nitro-tyrosine's sites were identified.

Electron Spin Resonance (ESR) analyses

Electron Spin Resonance (ESR) analyses were performed on
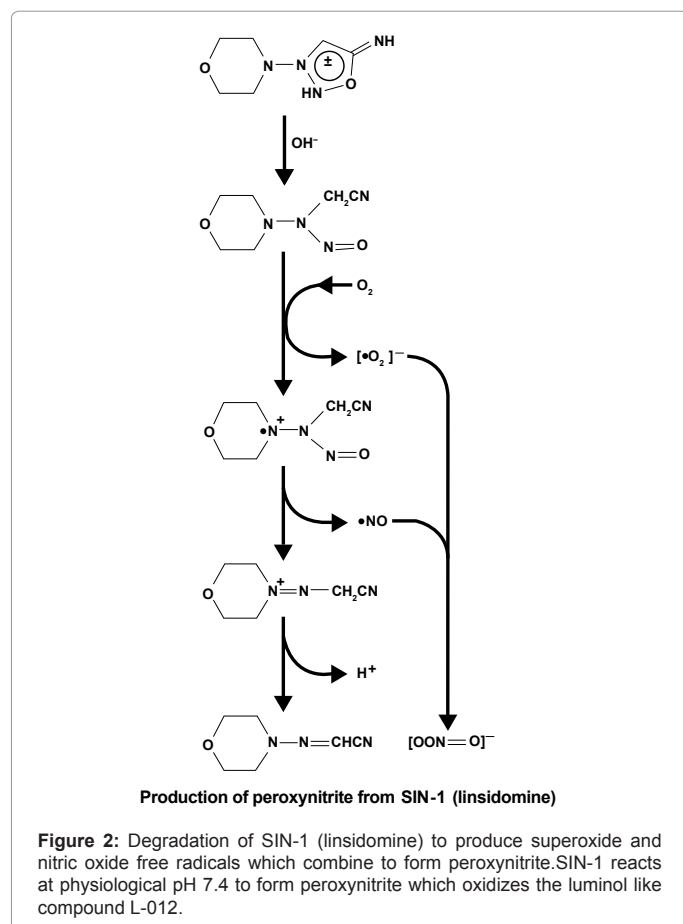
five separate solutions in a Brucker EMX electron spin resonance instrument. The first ESR analysis consisted of a carboxy-PTIO solution diluted with PBS pH 7.4. The second solution was composed of diethylamine NONOate (DEA NONOate), a nitric oxide generator, added to a carboxy-PTIO diluted with potassium biphthalate buffer pH 4.0. A third solution containing streptozotocin in PBS, carboxy-PTIO, and potassium biphthalate buffer was examined also. A fourth ESR analysis consisted of a solution mixture containing streptozotocin in PBS, carboxy-PTIO, Cu^{++}, and ascorbate in potassium mixture biphthalate buffer. The fifth analyzed by ESR contained streptozotocin in PBS, carboxy-PTIO, Cu^{++}, and ascorbate in citrate in a potassium biphthalate buffer.

Nitration of acetaminophen

Peroxynitrite (1 μM) in 0.3 M NaOH was added to 1 μM acetaminophen and reacted at room temperature for five minutes. The solution was titrated to pH 7.4, diluted in methanol, and assayed using Anionic Direct Probe Mass Spectrometry (ADPMS).

Analysis of nitrated tyrosine damage from thin slices of diabetic and non-diabetic animals' eyes

Animals were sacrificed with pentobarbital, and blood and eyes were obtained from the rats for assay. Using standard techniques for mounting the eyes, a microtome was used to obtain equivalent slices (20 micron). Slices were incubated in physiological buffer containing rabbit antibodies specific for nitrotyrosine according to the manufacturer's specifications (Upstate Cell Signaling Solutions, Lake Placid, New York). The slices were incubated in a buffered solution containing anti-rabbit antibody labeled with horse rabbit peroxidase and assayed for luminescence using a 1:1 solution of a dilute luminol solution (0.1 MG %) and hydrogen peroxide (0.3%). Luminescence was read in Berthold 9505 C luminometer at 37˚C for five minutes (data not presented).

Slices mounted on slides were treated using a Tyramide™ Fluorescence Systems-signal amplification system (NEL701A) which contains streptavidin-horseradish peroxidase, a blocking agent, amplification diluent, fluorescein, and tyramide. We followed protocol II for in situ hybridization to assay for nitrotyrosine using the nitrotyrosine antibody identified above and then secondary labeling assay the TSF amplification kit. The green fluorescence of the tissue sections of the retinal tissue was read in a Leica fluorescence microscope using the appropriate filter displaying green at high intensity. A clear difference in nitrotyrosine of the diabetic eye tissue as compared to the non-diabetic-tri drug- treated eyes was evident. This observation of the difference between nitrotyrosine fluorescent labeling between diabetic and non-diabetic retinal slices has been reported previously by Kowluru in Diabetes [23].

Statistical analysis

All important quantitative experiments in this work were accomplished four times or more and the standard error of the mean was calculated to evaluate the variation from the mean between groups. The student's *t* test for significance was applied between like groups to determine the level of significance. A confidence level set at (95%) was considered significant i.e. p=0.05. The differences between tri drug/STZ treated animals and the diabetic STZ –treated animals was so large as to reveal significance almost by inspection alone. The actual p values for the different experiments involving triple- drug STZ animals and the STZ-treated animals were below 0.01. The reason that this happened was because Duran-Reyes et al used excessive STZ and not enough of the nitric oxide converter carboxy-PTIO. By using less STZ and more
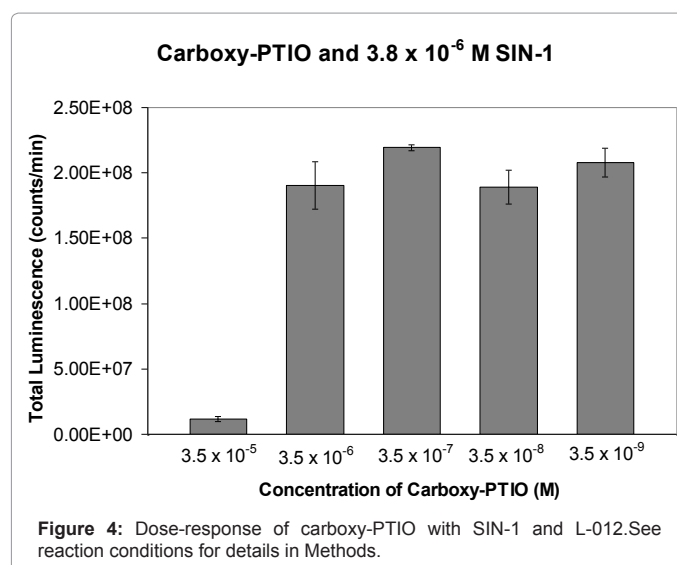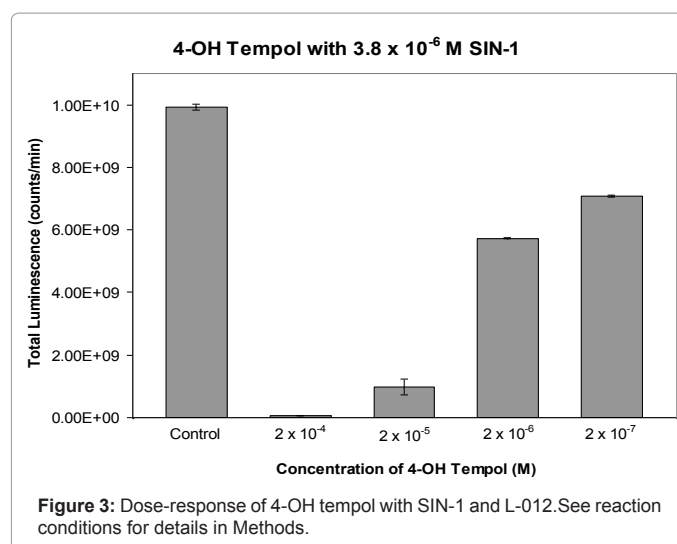
c-PTIO in our work, we believe that the nitric oxide generated inside the pancreatic β cell was completely converted to NO_2 radical and thus not converted to the NO-based toxin thus preventing the diabetes!

Results

The degree of inhibition of the luminescent reaction between SIN-1 (peroxynitrite generator) and L-012 by various concentrations of 4-OH tempol is shown in Figure 3. Because tempol chemically converts superoxide to hydrogen peroxide, it inhibits the generation of peroxynitrite which is dependent on the simultaneous production of superoxide and nitric oxide. Inhibition of oxidation of L-012 occurs between 2×10^{-4} M to 2×10^{-7} M 4-OH tempol.

The reaction between carboxy-PTIO and SIN-1 with L-012 is shown in Figure 4. Carboxy-PTIO is a scavenger of free nitric oxide. However, carboxy-PTIO does not inhibit the luminescence reaction between SIN-1 and L-012 at doses less than 0.2 μM.

The inhibition by acetaminophen of the SIN-1 and L-012 luminescent reaction occurs at concentrations ranging between 4×10^{-5} M to 4×10^{-9} M in Figure 5.

Figure 3: Dose-response of 4-OH tempol with SIN-1 and L-012. See reaction conditions for details in Methods.

Figure 4: Dose-response of carboxy-PTIO with SIN-1 and L-012. See reaction conditions for details in Methods.
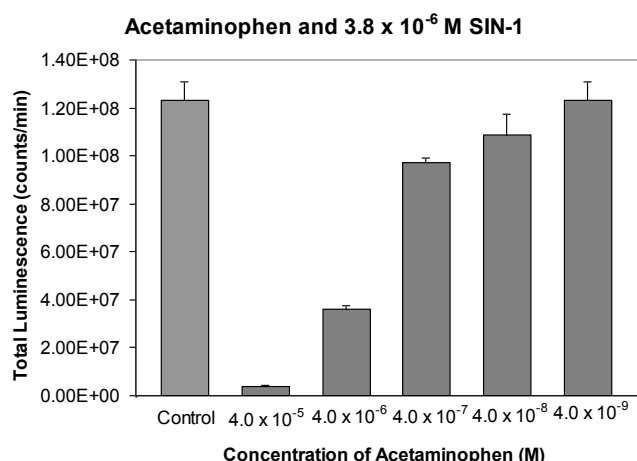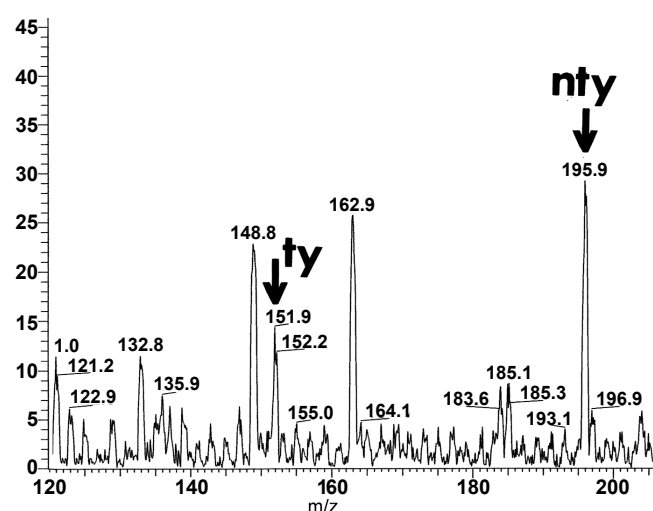
The mass spectrum of acetaminophen and nitro-acetaminophen showing that acetaminophen can be nitrated by peroxynitrite (from SIN-1) is shown in Figure 6.

In Figure 7 the difference in the appearance of an acetaminophen solution (water clear) and a nitro-acetaminophen solution (yellow) can be readily appreciated.

The electron spin resonance pattern of carboxy-PTIO (five peaks) at pH 7.4 and carboxy-PTIO reacted with DEA NONOate (seven peaks) at pH 4.0 are shown in Figure 8. This indicates that carboxy-PTIO reacts with free nitric oxide generated by DEA NONOate, a nitric oxide generator. Carboxy-PTIO and streptozotocin when reacted together at pH 4 produce five peaks indicating little nitric oxide is generated where seven peaks should be seen. In the presence of Cu^{++} and ascorbate seven peaks are seen indicating possible importance of oxidation. When combined with citrate only two ascorbate radical peaks are seen showing chelation of the copper ion stops the oxidation. This may indicate oxidation inside beta cells may be necessary to produce nitric oxide free radicals.

Figure 5: Dose-response of acetaminophen with SIN-1 and L-012. See reaction conditions for details in Methods.

Figure 6: Mass spectrum (ionization detector) of acetaminophen and 3-nitroacetaminophen.
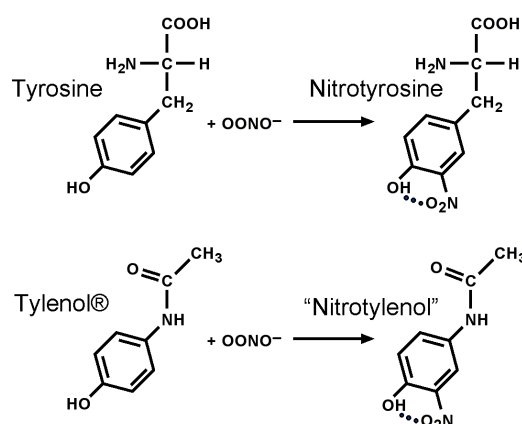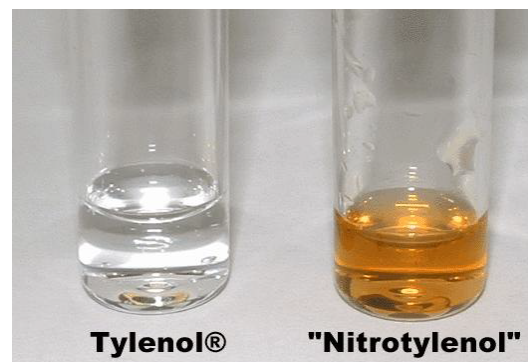
**Figure 7:** Photograph of a buffered solution of acetaminophen and 3-nitroacetaminophen.

The non-denaturing electrophoresis PAGE gel pattern of insulin nitration is shown in the upper Figure of Figure 9 A. The effect of acetaminophen, catechin, green tea, and quercetin (mono and polyphenols) on the nitration of insulin is clearly demonstrated. The lower Figure 9B shows the number of nitrosylated groups per insulin molecules analyzed by densitometry. The nitration of insulin was accomplished using an alcoholic solution of tetranitromethane. The degree of nitration averaged about 2-3 nitro groups per insulin molecule. When a mixture of nitroinsulin (2.8 nitrotyrosines/insulin) was assayed for its anti-diabetic action, it was found to be 50% active. Green tea and quercetin (polyphenols) inhibit nitration best, but acetaminophen and catechin (monophenols) also somewhat effective in inhibiting insulin nitrosylation. Using a less active nitrosylating system (SIN-1) acetaminophen at 100 micromolar reduces the average degree of nitration of insulin to one nitro group/insulin, a nitration level that does not produce a clear decrease in insulin glucose lowering activity in diabetic animals.

The MS/MS mass spectrum pattern of the insulin β-chain digested using a trypsin-proteolytic-enzyme digestion as shown in Figure 10.

The MS/MS mass spectrum pattern of tetranitroinsulin is shown in Figure 11, while Figure 12 identifies the tyrosines nitrated (yellow) in the tetranitrated insulin molecule.

The blood glucose levels seen in streptozotocin treated rats (with and without Triple Drug Treatment (TCH)) are shown in Figure 13.

The eyes of 6 rats injected with streptozotocin (cataracts) or
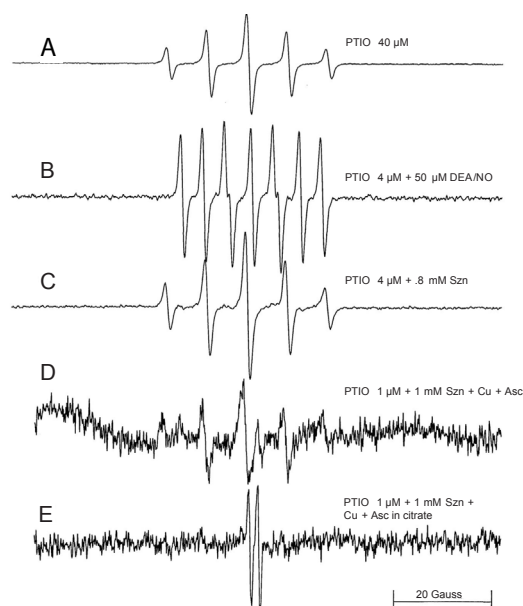
Figure 8: The electron spin resonance patterns of carboy-PTIO (sodium salt) alone and various inhibitors and/or conditions.Carboxy-PTIO alone generates five peaks as seen in Figure 8A.When DEA-NONOate (DEAN) is added to the carboxy-PTIO, seven peaks are generated due to the reaction from nitric oxide (coming from DEAN) and carboxy-PTIO (see Figure 8B). Carboxy-PTIO and streptozotocin generates mostly five peaks indicating little NO.generation (see Figure 8C).In Figure 8D Copperion (Cu^{++}) is added to the reaction in 8D only two radical ascorbate peaks are seen.

Figure 9: A) PAGE electrophoresis (natural) of human insulin and nitrated insulin with and without inhibitors.Nitration of insulin was accomplished in 80% ethanol using dilute 1:100 tetranitromethane.The inhibitors of nitration were catechin, acetaminophen, quercetin and green tea extract.See Methods for details.Ten milligrams of insulin or nitroinsulin were spotted. Nitration varied from 2.0 to 2.8 nitro groups per insulin on average.However, Commassieblue staining indicates 4 or 5 nitroinsulins produced.Each insulin has four total tyrosines that can be nitrated (two in alpha chain and two in beta chain). B) Densitometry of the Comassie stained gel from the PAGE electrophoresis gel was accomplished with a Typhoon 9400 and analyzed with its software.
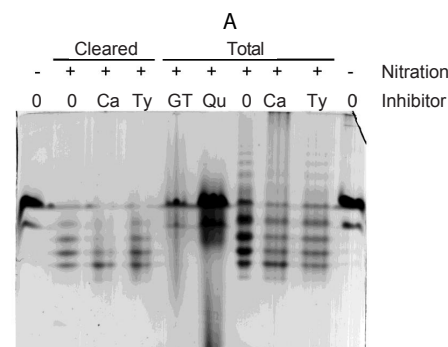
simultaneous streptozotocin and triple drug combinations (sodium carboxy-PTIO, 4-OH tempol, and acetaminophen) six months post injection (no cataracts) are shown in Figure 14.

The difference between nitrotyrosine-fluorescent labeling of control and diabetic rats' eyes is shown in Figure 15 A and B. The green fluorescence due to nitrotyrosine labeling seen in non-diabetic and diabetic retinal slices is depicted, and the amount of fluorescence is qualitatively more in the retina observed in diabetic rats versus non-diabetic rats.
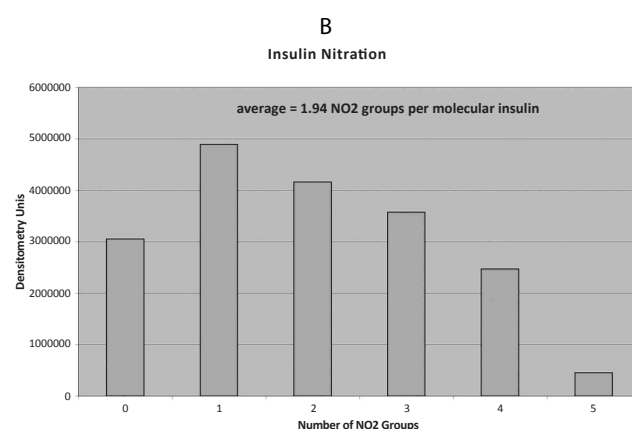
Discussion

In both Type I and eventually diabetes Type 2 there can be destruction of the pancreatic β cells and subsequent loss of insulin synthesis and secretion (1). Research in animal models [3,4,10-22] has suggested that nitric oxide (NO.) and superoxide (.O$_2$)$^-$, NO$_2$, N$_2$O$_4$ or peroxynitrite (OONO$^-$) may play a role in the pathogenesis of type I or type 2 diabetes via oxidation or nitration based on toxicity mechanisms.

A simple model to study the interaction of superoxide, nitric oxide, and peroxynitrite with other molecules is the reaction between SIN-1 (peroxynitrite generator) and luminal [24] or the luminol derivative, L-012 [14]. The generation of peroxynitrite from SIN-1 is illustrated in Figure 2.When SIN-1 degrades in a basic solution, superoxide is generated which reacts with nitric oxide on the surface of the degrading molecule producing peroxynitrite (OONO$^-$). Peroxynitrite then oxidizes L-012 (a luminol-like molecule) producing a blue light or luminescence. Substances like mono- or polyphenols can act as antioxidants and interfere with the oxidation of L-012 thereby inhibiting the production of blue light [14].

It is well known that the 4-OH tempol free radical can react with superoxide (.O$_2$) - and produce hydrogen peroxide (H$_2$O$_2$). This tempol inhibits the luminescence of L-012 of the SIN-1 (peroxynitrite reaction) [3]. This is shown in this reaction in Figure 3 in which tempol inhibition and at concentrations ranging from 2×10^{-4} M to 2×10^{-7} M. Because tempol reacts with superoxide generating hydrogen peroxide, it deprives the nitric oxide radical of its necessary partner and inhibits the generation of peroxynitrite (OONO$^-$) [3] and the subsequent peroxynitrite oxidation of the L-012 to produce blue light is markedly inhibited which is detected by the luminometer.

One would expect the free radical carboxy-PTIO to inhibit the luminescent reaction of SIN-1 and L-012.Moreover, carboxy-PTIO reacts with the free radical nitric oxide to produce a NO$_2$ free radical [10,21,22]. However, carboxy-PTIO inhibits luminescence of SIN-1 and L-012 only at a concentration of 2×10^{-5} M (Figure 4). Sequential 10 fold dilutions of carboxy-PTIO show no evidence of inhibition. This is likely due to the fact that the carboxy-PTIO reaction with nitric oxide is much slower than the reaction of nitric oxide and superoxide produced from SIN-1 which reacts at diffusional speed of 10^9 second.
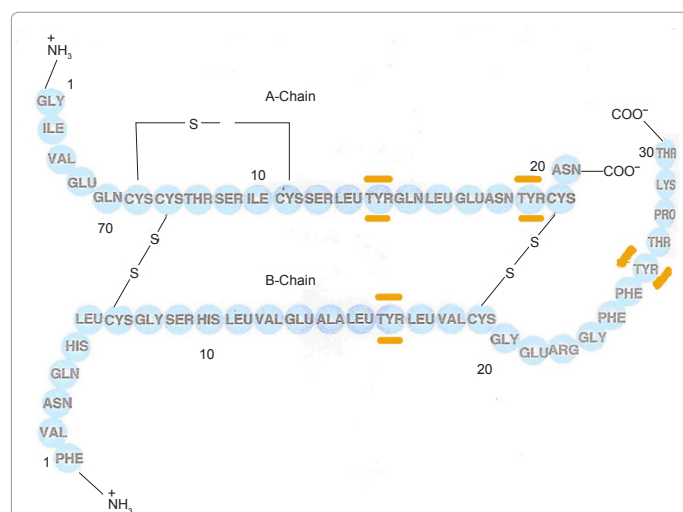
Acetaminophen inhibits the luminescence reaction between SIN-1
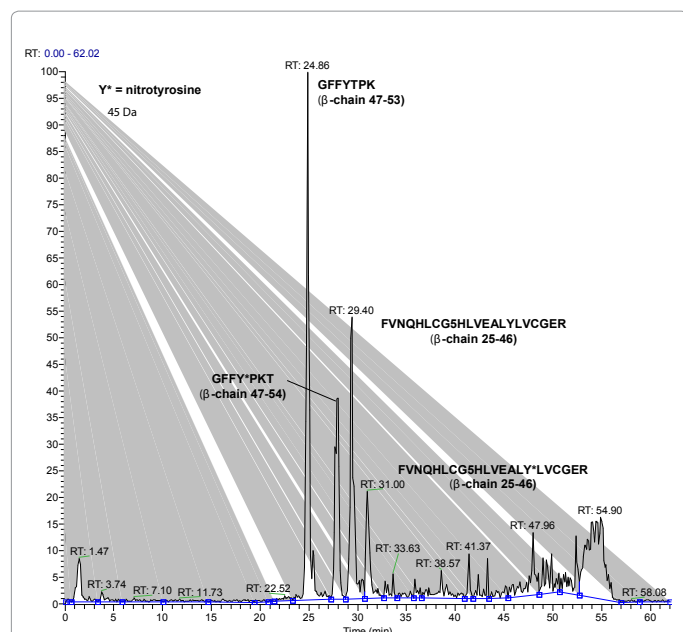
Figure 10: ms/ms spectrum of the beta chain of nitrated human insulin digested using trypsin proteolysis.See Methods for details.
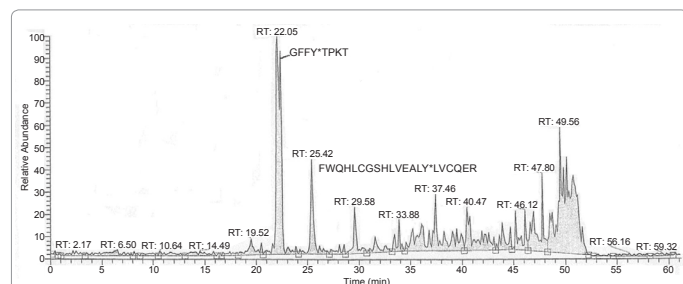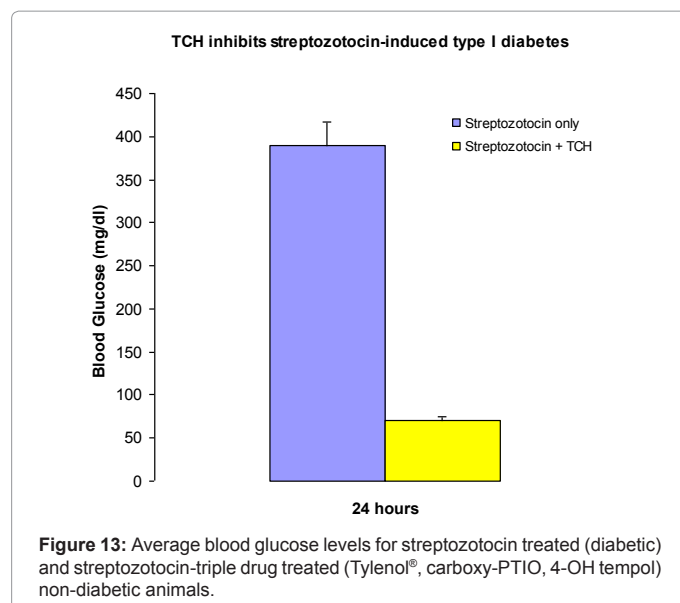

**Figure 11:** Mass spectrum of tetranitroinsulin. See Methods for details.

Figure 12: Human insulin molecule demonstrating the tyrosines that may be potentially nitrated.

and L-012 in a dose-responsive manner from 2×10^{-4} M to 2×10^{-8} M (Figure 5). Acetaminophen can be nitrated by SIN-1 (Figure 6) and the

Figure 13: Average blood glucose levels for streptozotocin treated (diabetic) and streptozotocin-triple drug treated (Tylenol®, carboxy-PTIO, 4-OH tempol) non-diabetic animals.
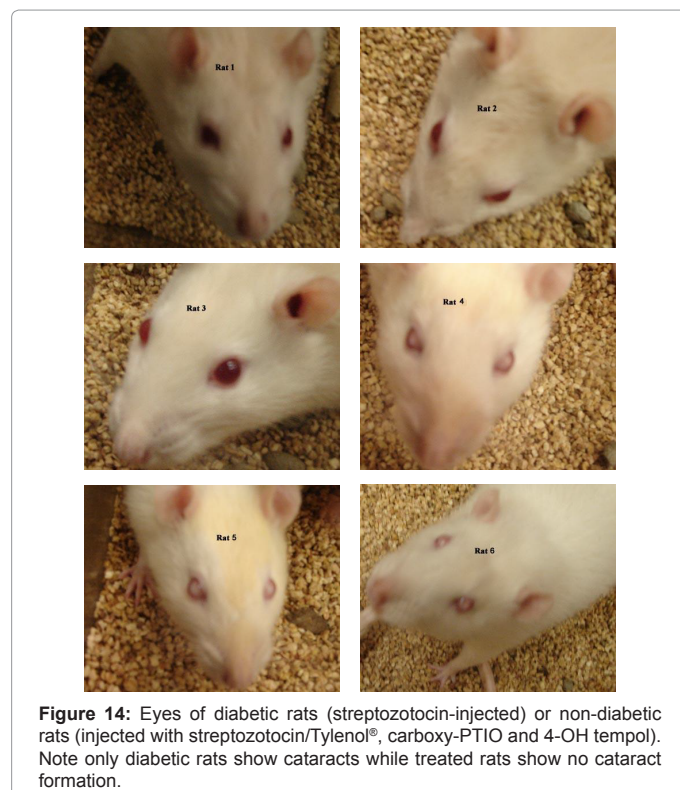
Figure 14: Eyes of diabetic rats (streptozotocin-injected) or non-diabetic rats (injected with streptozotocin/Tylenol®, carboxy-PTIO and 4-OH tempol). Note only diabetic rats show cataracts while treated rats show no cataract formation.

acetaminophen turns yellow (Figure 7) and results as an antioxidant of L-012.

The acetaminophen mass spectrum M/Z is displayed in Figure 6 at 151.9 mass while that of acetaminophen is at 195.9 mass. The actual solutions of acetaminophen and nitro-acetaminophen are seen in Figure 7. In addition, the chemical similarity between the nitration of tyrosine and the nitration of acetaminophen is seen as hydrogen bonded structures which cause an intense yellow color due to the increased chemical resonance of the nitrated structure.
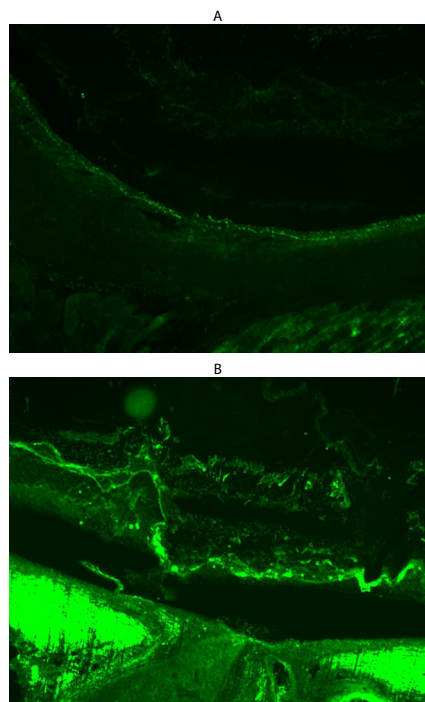
Figure 15: A) Nitrotyrosine labeled retinal slices using TSA histochemical fluorescence of the retina of atri-drug /STZ treatednon- diabetic rat. B) Slice of retina from a STZ diabetic rat.A TSA- fluorescence enhancer system was used and photographed using a fluorescent microscope.See Methods for details.

The fact that tyrosine and acetaminophen are similar monophenols that can be readily attacked via nitration has a possible new meaning in diabetes. If nitric oxide, NO_2, N_2O_4, and peroxynitrite are generated in diabetes as indicated by increased nitration of proteins in the diabetic state [24], the possibility that insulin itself can be nitrated in one or more of its four tyrosine's in the diabetic state must be raised. In addition, insulin receptors or insulin like proteins are known to contain tyrosine's that could be nitrated and present themselves as competitors to insulin producing an insulin resistance-like state depicting phenols inhibiting nitration of insulin (Figure 9A). Substances such as acetaminophen, quercetin, or green tea polyphenols could protect insulin or other proteins by inhibiting the nitration or oxidation of their tyrosines moieties [24].

Free radical carboxy-PTIO can be detected using Electron Spin Resonance (ESR)-reacted for 20 minutes. This is shown in Figure 8A which depicts the five line electron spin resonance spectrum of carboxy-PTIO. When the carboxy-PTIO is combined with DEA NONOate, a nitric oxide generator, it produces a seven line ESR splitting pattern. This seven line splitting pattern is due to the loss of oxygen from carboxy-PTIO, which oxidizes the NO. Generated by the NONOate to form carboxy-PTI and NO_2 (Figure 8B). The seven line pattern signifies the non-symmetrical intermediate prior to formation of the PTI radical. However, when carboxy-PTIO is combined with streptozotocin at pH 4.0, it generates a five line ESR with a minor seven line pattern, similar to the ESR generated by the carboxy-PTIO alone. The five line ESR generated by the carboxy-PTIO and streptozotocin (Figure 8C), is similar to the ESR generated by carboxy-PTIO alone. The combination of carboxy-PTIO and streptozotocingenerate a five line ESR with small peaks present between the five larger lines,

suggesting that a small amount of NO. may be present, but not enough to generate a significant amount of carboxy-PTI (which would produce a seven line ESR pattern).Since streptozotocin is a nitric oxide generator, one would anticipate that a seven line ESR would be generated. The conditions present in the ESR tube may have not created a favorable enough environment for streptozotocin to produce enough nitric oxide to produce the seven line pattern. Flodstrom et al. [21] reported that mice lacking NO synthase II, when given streptozotocin, do not produce nitric oxide. Therefore, NO synthase as well as other components in the beta cell may be necessary to allow the production of nitric oxide from streptozotocin. NO synthase contains heme and zinc which may enhance NO· production. When Cu^{++} and ascorbate are added to streptozotocin and carboxy-PTIO, seven lines are produced as in Figure 8D.This finding reproduces the work of Tsuji and Sakurai [13]. However when citrate is added to the previous mixture, only ascorbate radicals are seen showing oxidation has occurred (Figure 8E).

It has been shown that insulin can be nitrated using SIN-1 or peroxynitrite. However, complete nitration of all tyrosines is difficult using this chemistry. The average nitration is less than one nitro group per insulin molecule. Our approach to this problem was to use the highly active nitrating system of tetranitromethane in an alcoholic solution [25]. This produces insulin with an average of 1.8 to 2.8 nitro groups per insulin molecule. Then, polyacrylamide-non-denaturing-gel electrophoresis can be used to separate the multi-nitrated insulin species since each tyrosine nitrated produces a negative charge. The gels can be stained using Comassieblue and then partially destained to promote better contrast to the heavily stained insulin species (Figure 9A). A densitometry scan of the separated nitro insulins using a GS-800 Calibrated Densitometer (BioRad) is easily accomplished (Figure 9B). In addition to nitrating insulin alone, the nitration of insulin can be partially inhibited with a competitor for inhibition such as acetaminophen, catechin, quercetin or green tea extract. Any mono or polyphenol having aromatic chemistry open to nitration might inhibit the nitration of insulin. The polyphenols in green tea or the polyphenol quercetin when added at higher concentrations than acetaminophen or catechin are even effective inhibitors of insulin nitration. When the dose of acetaminophen is increased to a millimolar level, SIN-1 nitration of insulin is inhibited to a value of one nitro group/insulin molecule. Therefore, the dose of the polyphenol is crucial to the inhibition of nitrosylation of tyrosines in proteins. Chi Q et al. [26] have shown mononitrated insulin has little ability to reduce glucose levels in diabetic rats. However, the crude 2.8 nitro group/insulin molecule species reduced the glucose lowering effect of the insulin by 50% in diabetic rats. This heavily nitrated insulin's lose most of their glucose lowering capacity suggesting multiple free tyrosines in the insulin peptides are necessary to be present for the full glucose lowering effect. This may mean that free tyrosines of insulin are necessary to bind to the insulin receptor *in vivo* to produce the sugar lowering effect of insulin in an animal or human.

Mass spectrometry using MS/MS analysis of proteolytically digested β-chain of nitrated insulin indicates nitration of both tyrosines of the β-chain (Figures 10 and 11). See mass spectroscopy of the two tyrosines which can be nitrated in peptides A and B. The amino acid sequence of the two peptide chains that comprise the structure of insulin can be seen in Figure 12 with the tyrosines labeled in yellow.

The blood glucose levels from Streptozotocin (STZ) injected animals is shown in Figure 13 compared to a group of animals injected with a STZ + tri-drug combination the latter group of treated animals have glucose concentrations similar to normal (non-diabetic rats)

(Figure 13). The tri-drug combination can prevent type I diabetes as judged by the four fold or greater difference between the glucose levels of the diabetic versus treated animals. Basically once the tri-drug combination protects against damage by a single injection of STZ, all animals are diabetes free their entire life.

Nitric oxide appears to play a role in oxidative damage located in the eye, leading to diabetic cataracts [27]. All tri-drug combination/ STZ animals of Figure 14 at 3 months and later have no cataracts and the five diabetic (STZ only) animals all have cataracts. Clearly the cataracts are the result of the streptozotocin diabetic associated injury which has been prevented by the tri-drug combination.

The enhanced fluorescence linked to nitrotyrosine in STZ-diabetic eyes versus tri-drug/STZ treated non-diabetic eyes is shown in Figure 15A and B. This Figure clearly demonstrates a qualitatively greater amount of the nitration associated with diabetes. All of the data presented suggest that oxidative and nitrosative stress caused by excessive glucose levels are central for glucose induced toxicity and the various complications associated with diabetes [28]. Robertson [4] has identified six different biochemical pathways by which excessive glucose leads to increased Reactive Oxygen Species (ROS) which is responsible for these complications associated with diabetes mellitus. He has suggested use of vascular multi antioxidant drugs as a protective mechanism e.g. N-acetyl-cysteine, metformin and troglitazone as well as vitamin E can alleviate some of the variety of these complications.

The nitration of tyrosines in protein can profoundly affect the activity of enzymes and/or protein hormones. Nomiyamia et al. [29] have shown that tyrosine nitration of the insulin receptor substrate-1 reduces insulin stimulated glucose uptake. Smith et al. [30] have shown that peroxynitrite-mediated tyrosine nitration inhibits superoxide dismutase which limits the concentration and half-life of superoxide necessary to generate peroxynitrite (OONO-) from its reaction with nitric oxide. Zou M et al. [9] demonstrated an inactivation of prostacylin synthase by tyrosine nitration. There are many other examples of this because tyrosine is found in the active sites of many enzymes, critical proteins and hormones.

It is well known that diabetes causes a chemical modification of proteins via glycosylation, oxidation and nitration [31]. These altered proteins are in blood and urine. If key molecules e.g. proinsulin insulin, insulin receptor substrates 1 and 2, other insulin receptors or insulin like growth factor I or II are nitrated *in vivo* they could play an important role in the pathogenesis of insulin resistance involved with type 2 diabetes.

Recent data from Unger and Cherrington [2] indicates that when the beta cells die, alpha cells take their place and produce excessive glucagon peptide which raises blood sugar. Therefore, diabetes I occur in part due to an imbalance in the glucagon/insulin ratio which can be corrected by replacing insulin and/or inhibiting or depleting glucagon. It is well known that a major symptom in diabetes 2 is insulin resistance. In addition type 2diabetics display a greater degree of cataracts than individuals with type 1 diabetes. In our model all animals not protected by carboxy-PTIO but injected with STZ developed cataracts in three months post injection. After our experiments were finished we found the recent work of a Chinese group, that uses similar doses of STZ but given twice and they added a high fat diet [31]. The pathology in their animal's resembled type 2diabetes, but they failed to mention the development of cataracts. Possibly this was due to the fact that their experimental animals were not used for extended periods of time post STZ injection.

Conclusions

Would this combination of agents be effective in human diabetes? If the chemistry responsible for β cell destruction in animals is similar to that occurring in man, the tri-combination might be helpful. However, it is not currently possible to know when early type I diabetes occurs until 80-90% of β cells are gone and overt diabetes mellitus is evident [1,4]. Whether a combination of carboxy-PTIO, 4-OH tempol and acetaminophen (Tylenol®) can prevent type I or type 2 diabetes mellitus at a later stage of diabetes mellitus development is as yet unclear. Moreover, whether the use of 4-OH tempol and carboxy-PTIO would be safe if used over time in humans has never been tested. But the concepts developed in this manuscript suggest that if treated early in the disease state, diabetes is a treatable if not curable disease. However, this is very dependent on developing an inexpensive high volume screening system for the Type 1 disease. We are currently developing such a test in our laboratory to recognize the early vulnerability of certain children and even young adults to the ravages of this disease so that this diabetes can be prevented!

The treatment of early type 2 diabetes is much easier than type 1 since blood glucose can be easily monitored early in the disease and drugs for type 2 utilized and/or control of body weight via diet can done alone or concurrently with drug treatment.

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