

## **Advanced glycation end-product pentosidine accumulates in various tissues of rats with high fructose intake**

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Short title: Pentosidine accumulates in various tissues of rats

### **Summary**

The slowly metabolized proteins of the extracellular matrix, typically collagen and elastin, accumulate reactive metabolites through uncontrolled non-enzymatic reactions such as glycation or the products arising from the reaction of unsaturated long chain fatty acid metabolites (possessing aldehydic groups). A typical example of these non-enzymatic changes is the formation of advanced glycation end-products (AGEs), resulting from the reaction of carbohydrates with the free amino group of proteins. The accumulation of AGEs and the resulting structural alterations result in altered tissue properties (increased stiffness, reduced elasticity) that contribute to their reduced susceptibility to catabolism (diabetes mellitus) and to their aging. Posttranslational nonenzymatic modifications of the proteins of the extracellular matrix (the formation of a typical AGE product - pentosidine) were studied in three types of tissue of three types of rats subjected to a high fructose diet. Chronic (three-week) hyperglycemia (resulting from fructose loading) caused

a significant increase in pentosidine concentration mainly in the aortas and skin of the three rat strains (Lewis, Wistar and hereditary hypertriglyceridemic (HTG) rats).

**Key words:** collagen, pentosidine, AGEs, elastin, glycation

## **Introduction**

Proteins undergo important non-enzymatic (chemical) posttranslational modifications with a significant physiological/biological impact. The most susceptible proteins are the slowly metabolized ones, having a longer exposure time for chemical reactions with endo- and exogenous compounds (Maillard 1912; Trivelli *et al.* 1971).

The extracellular matrix is formed by these slowly metabolized proteins, typically collagen and elastin. One of the typical non-enzymatic reactions is glycation, the reaction between the reactive oxo-group of the sugar and amino group of the protein (Deyl and Miksik 1997; Miksik and Deyl 1997; Monnier 1989; Monnier *et al.* 1992). In this sequence of reactions, a number of compounds arise that are not yet completely known, including monotopically as well as multitopically (cross-link) bound products. This series results in the formation of advanced glycation end-products (AGEs). The accumulation of AGEs and the resulting structural alterations result in altered tissue properties (higher stiffness, lower elasticity) that contribute to their reduced catabolism and to their aging. Collagen, which is a typical long-life protein, loses flexibility and enzymatic digestivity in parallel with aging (Schnider and Kohn 1981). Similar changes in collagen are known to occur in diabetic patients (Schnider and Kohn 1982). AGEs in mammals have been predominantly chemically characterized by the detection of fluorescent products – namely pentosidine (imidazo [4,5-b] pyridinium molecule comprising a lysine and arginine), one of a few presently known glycation products (Sell and Monnier 1989).

One of the most globally prevalent metabolic syndromes, diabetes mellitus, is a major risk factor for cardiovascular disease, and atherosclerosis accounts for most of the morbidity and mortality of

diabetic patients. Diabetes mellitus is characterized by hyperglycemia, resulting in metabolic changes in lipid and protein metabolism and irreversible long-term vascular and connective tissue changes. These changes include diabetes-specific complications such as retinopathy, nephropathy and neuropathy and complications of the macrovasculature such as atherosclerosis, potentially resulting in heart disease, stroke and peripheral vascular disease (Hudson *et al.* 2001). AGEs in hyperglycemic patients are reviewed in (Hatfield and Mulfinger 2005). Pentosidine has been found to accumulate in the aorta, tail and skin collagen matrix at accelerated rates in diabetic mammals (Kern and Engerman 2001; McCance *et al.* 1993; Nyengaard *et al.* 1997). Links between chronic hyperglycemia and the development of long-term diabetic-specific complications have been discovered but not yet completely understood (Hudson *et al.* 2001; Reddy 2004; Wautier and Guillausseau 2001).

The passive biomechanical properties of vessels change with age. An increase in thickness and stiffness, as well as a reduction in elasticity of the aorta during aging have been shown in both rats and humans (Bruehl *et al.* 1998; Bruehl and Oxlund 1996; Sonesson *et al.* 1997). These changes are primarily caused by smooth muscle cell hypertrophy and fibrosis. During aging and senescence, the concentration of collagen and elastin changes in the rat aorta (Bruehl and Oxlund 1996). The elastin concentration decreases and the concentration of collagen (mainly collagen type III) increases up to a certain age and then reaches a plateau (Cox 1983; Cox and Detweiler 1988; Vogel 1991).

Cardiovascular diseases are in many cases accompanied by lipid disorders such as obesity (Marin-Garcia and Goldenthal 2002). Arterial hypertension is a widely studied problem with a direct relationship to modification of the vessel wall. Hypertension often results in an increased thickness and stiffness of large artery walls (Clerc *et al.* 1999; Karam *et al.* 1996; Laurent 1995; Laurent *et al.* 1994). The AGEs of plasma and matrix proteins are candidate mediators for various vascular complications such as atherosclerosis. A significantly larger accumulation of AGEs was detected in the aorta of stroke-prone, spontaneously hypertensive rats (Mizutani *et al.* 1999).

In this study, hereditary hypertriglyceridemic (HTG) rats were used, which represent a genetic model of hypertension causing metabolic disturbances in lipid and carbohydrate metabolism (Stolba *et al.* 1992). This animal model seems to be suitable for studying the posttranslational products arising from both reactions with reducing sugars and with lipid-derived compounds. HTG strain of rats was used therefore we assumed theoretic chance of influence of aldehydic intermediates of lipids to the formation of the AGEs namely pentosidine. The pentosidine concentration in the skin and tail tendon collagen of HTG rats significantly increases with age (Miksik *et al.* 1997).

The posttranslational non-enzymatic modifications of extracellular matrix proteins (the formation of the typical AGE - pentosidine) were studied in three types of tissues (skin, tail tendons and aortas) of three strains of rats (Wistar, Lewis and HTG) that were also subjected to a high fructose diet.

## **Methods**

### **Chemicals**

All chemicals used were of either analytical grade or the highest available purity. Trifluoroacetic acid and acetonitrile (HPLC gradient grade) were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma (St. Louis, MO, USA). All solutions were prepared in Milli-Q Water (Millipore, Bedford, MA, USA).

### **Instrumental**

The HPLC apparatus used was a Agilent 1100 (Agilent, Fullerton, CA, USA) consisting of a degasser, a binary pump, a thermostated column compartment, diode array and fluorescence detectors; the column used was an Atlantis (150 x 3.9 mm I.D., 3  $\mu$ m, C<sub>18</sub>, Waters, Ireland).

### **Experimental animals**

Three strains of rats were used:

- a) six month-old male HTG rats. The HTG rats were originally selected from the Wistar rats as previously described (Vrana and Kazdova 1990).
- b) six month-old male Wistar rats
- c) six month-old male Lewis rats

All three strains of rats were split to two groups:

- 1) control groups (C) – were fed a standard rat chow.
- 2) groups kept on high fructose diet (10% F) – the rats drank 10% fructose solution for the last three weeks before being sacrificed.

Every group contained at least five animals.

#### Sample preparation

Three types of tissue samples: a) skin, b) tail tendons, and c) aorta were taken from all the groups of rats.

All samples were prepared according to a previously published procedure (Dunn *et al.* 1991). Briefly, after thawing, the skin samples were scraped vigorously with a scalpel to remove the adherent fat and other tissues (tendon and aorta samples were prepared without this step). All samples were extracted sequentially for 72 h at room temperature with chloroform-methanol (2:1), and 1 mol/l NaCl, to remove the lipids and soluble proteins. After the final extraction, the samples were rinsed in distilled water and dried by lyophilization.

#### Preparation of the pentosidine standard

The pentosidine standard was prepared by a modified method of Dyer *et al.* (Dyer *et al.* 1991). The pentosidine was purified in two subsequent separation steps by HPLC in a Supelcosil LC-318 column (250 x 4.6 mm i.d., 5  $\mu$ m, 300 Å pores). In the first step, separation was achieved by gradient elution using a water-acetonitrile gradient (with 0.1% TFA). In the second step, TFA was

replaced by 0.1% HFBA. The purified product was deacetylated by heating in 6 M HCl for 1 h at 110 °C, the pentosidine was finally purified using a system with 0.1% HFBA. The identification of pentosidine was confirmed by measuring its fluorescence spectra.

#### Amino acid analysis

The method uses precolumn derivatization with phenylisothiocyanate (the PICO-TAG system; Waters, following the manufacturer's instructions) followed by the separation of the resulting products by HPLC in a reversed-phase column (C<sub>18</sub>; Pico-Tag column 300 × 3.9 i.d. mm; Waters) using an acetate buffer (pH 6.4, 0.14 mol/l)–acetonitrile gradient (following the manufacturer's procedure).

#### Collagen and elastin assessment

Collagen and elastin levels were assessed from the amino-acid contents of glycine (about 33% of the residues in collagen and elastin), hydroxyproline (specific to collagen) and histidine (about 13% in elastin).

#### Pentosidine measurement

The hydrolysis of protein samples (skin, tail tendons and aorta) was carried out with HCl vapor (6 mol/l HCl with 2% phenol) for 20 h at 106 °C in a vial under vacuum. After lyophilization of 200 µl of hydrolysate, the sample was dissolved in 25 µl of 2% heptafluorobutyric acid (HFBA) and 10 µl was injected into the HPLC column (Atlantis: reversed-phase C18; 50 × 3.9 i.d. mm; Waters). Elution was done using a gradient of water with 0.1% HFBA (A) and acetonitrile with 0.085% HFBA (B) from 0% B to 9% B at 2 min, gradient continued from 9% to 17% at 21 min, and then from 17% to 100% B at 25 min. After that, the column was equilibrated with 100% A for 10 minutes. The flow rate was 0.8 ml/min and the column temperature was held at 40 °C. The

eluate was monitored by its fluorescence at 332ex/378em nm. The concentration of pentosidine was expressed as nanograms per milligram of total protein.

## Evaluation

All data are expressed as mean  $\pm$  standard deviation (SD). Statistical analyses of the data were performed and the probability values (P) for significance were calculated using Student's *t*-test. P values  $\leq 0.05$  were considered to be statistically significant.

## Results

The Amino-acid analysis of samples did not reveal any significant differences in the content of collagen and elastin between the controls and fructose-treated animals in the examined tissues of any of the rat strains (data not shown). Elastin content was higher (about 30 % of dry weight) in all aorta samples in comparison with skin and tail tendon tissues (in all rat strains) as expected, but there was no significant difference between the particular strains of rats.

In these experiments we used Wistar, Lewis and HTG rats that represent a genetic model of hypertension with accompanying metabolic disturbances of lipid and carbohydrate metabolism (Stolba *et al.*, 1992). It was assumed that these animal models are suitable for studying the posttranslational products arising from both the reaction with the oxo-groups of reducing sugars and with lipid-derived compounds.

In water drinking rats, the tissue concentration of pentosidine did not significantly differ among the three strains of rats (Wistar, Lewis, HTG) (Fig. 1). Under these conditions, the concentration of blood glucose was also not significantly different in these rat strains (Kadlecova *et al.* 2004). From this point of view, it was not surprising that the level of pentosidine, the compound arising from the reaction of sugar and amino acid, was not significantly different.

The situation was different when the rats were drinking 10 % fructose solution. The amounts of the posttranslational non-enzymatic product pentosidine significantly increased with high fructose

intake in all three strains of rats in the aorta and skin samples, and in the tendon sample of the HTG strain (Figs. 2, 3, 4). It should be noted that blood glucose levels were elevated by 60-80 % in fructose-drinking animals compared to the water-drinking controls (Kadlecova *et al.* 2004). The amounts of pentosidine in the tail tendons of fructose drinking Wistar and Lewis rats were higher than in the respective controls, but the variability of the concentrations was too high. In general, the pentosidine levels in tail tendons, aortas and skin of fructose-treated rats were highly variable.

## **Discussion**

This paper is focused on pentosidine – therefore it is only one known advanced glycation end-product crosslinking proteins, and for this reason it could have a strong influence on stiffness of the tissue. It could play a significant role first of all on blood vessels and it could be an additional risk factor for their normal function.

The amounts of pentosidine significantly increased with high fructose intake in all three strains of rats in the aorta and skin samples - our findings are in agreement with published results (Kern and Engerman 2001; McCance *et al.* 1993; Nyengaard *et al.* 1997), where pentosidine has been found to accumulate in the aortas of diabetic rats (Nyengaard *et al.* 1997) and dogs (Kern and Engerman 2001), in the skin of diabetic patients (McCance *et al.* 1993), and in the tail collagen of diabetic dogs (Kern and Engerman 2001).

The higher triglyceride content in the blood of HTG rats had no influence on the content of pentosidine in all three kinds of tendons. The changes in concentrations of pentosidine were not significant in a comparison of HTG versus Wistar, or HTG versus Lewis. From this finding we can conclude that the level of pentosidine is not dependent on the level of triglycerides and/or their metabolic products.

Any significant differences of the content of collagen and elastin between the controls and fructose-treated animals in the examined tissues of any of the rat strains were not revealed. We hypothesize that the high fructose intake had no influence on the ratio of these proteins. It is possible that ratio between collagen types (I and III) can change. This determination of the



collagen types is out of scope of this paper, but our group is preparing a new method (based on HPLC-MS measurement) is currently developing.

## Conclusions

HTG strain of rats was used therefore we assumed theoretic chance of influence of aldehydic intermediates of lipids to the formation of the AGEs namely pentosidine. But the skin, tail tendons and aortas of six month-old HTG rats had an unchanged extracellular matrix (as far as collagen and elastin content were concerned) and unchanged pentosidine content in comparison with Lewis and Wistar strain rats. No significant changes in the content of collagen and elastin were observed between the controls and rats kept on a high fructose intake. The higher triglyceride content in the blood of HTG rats had no influence on the content of pentosidine in all three kinds of tendons. Chronic (three-week) hyperglycemia caused a significant increase in the pentosidine concentration in the aorta and skin of three rat strains (Lewis, Wistar and HTG) and in the tail tendons of the Wistar strain. A higher pentosidine content was also observed in the tail tendons of Lewis and HTG rats, but these changes were not significant.

## Acknowledgments

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## Figure Captions

Fig. 1 Pentosidine content in control groups of rats: tail tendon, skin and aorta of HTG, Wistar, Lewis and HTG strains. Concentration is expressed as ng/mg of total protein.

Fig. 2 Pentosidine content of tissues of Lewis rats: controls and rats kept on water containing 10% fructose solution (10% F). Statistical significance: \* $P < 0.02$ . Concentration is expressed as ng/mg of total protein.

Fig. 3 Pentosidine content of tissues of Wistar rats: controls and rats kept on water containing 10% fructose solution (10% F). Statistical significance: \* $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$ . Concentration is expressed as ng/mg of total protein.

Fig. 4 Pentosidine content of tissues of HTG rats: controls and rats kept on water containing 10% fructose solution (10% F). Statistical significance: \* $P<0.05$ , \*\*  $P<0.02$ . Concentration is expressed as ng/mg of total protein.

Fig. 1

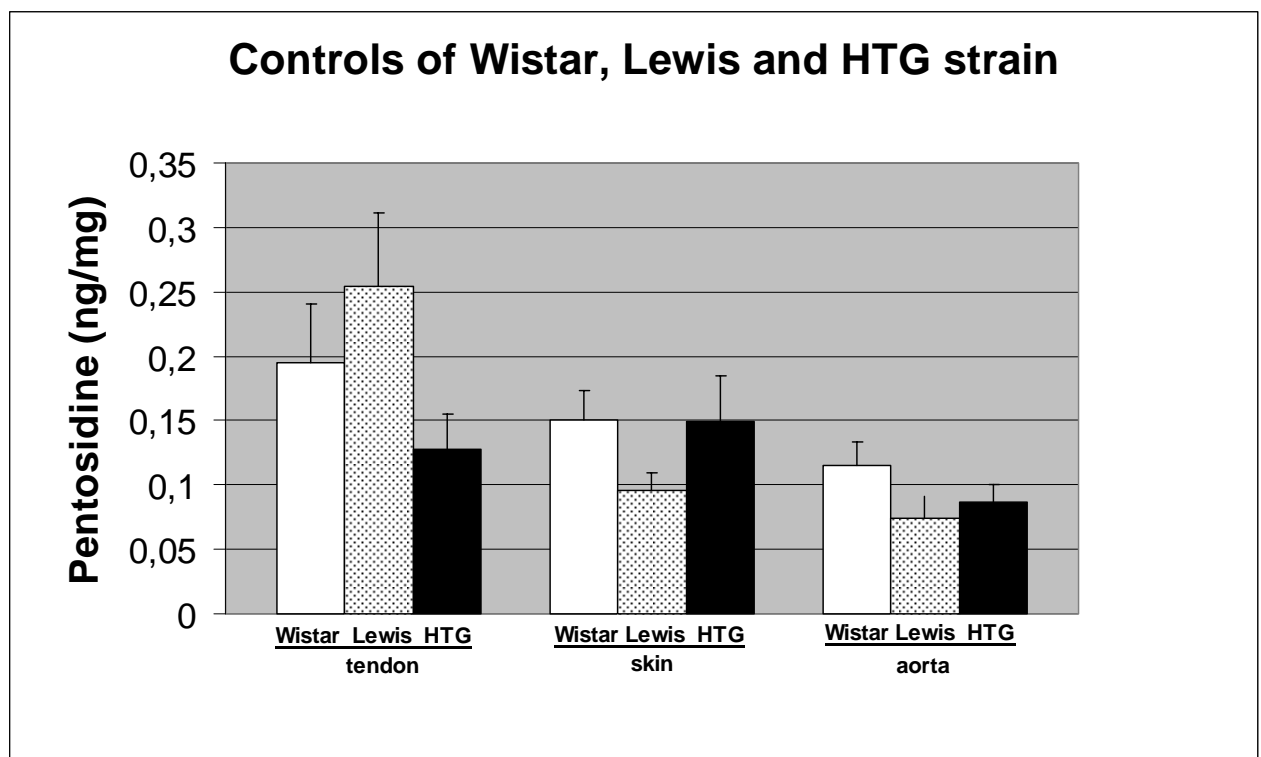


Fig. 2

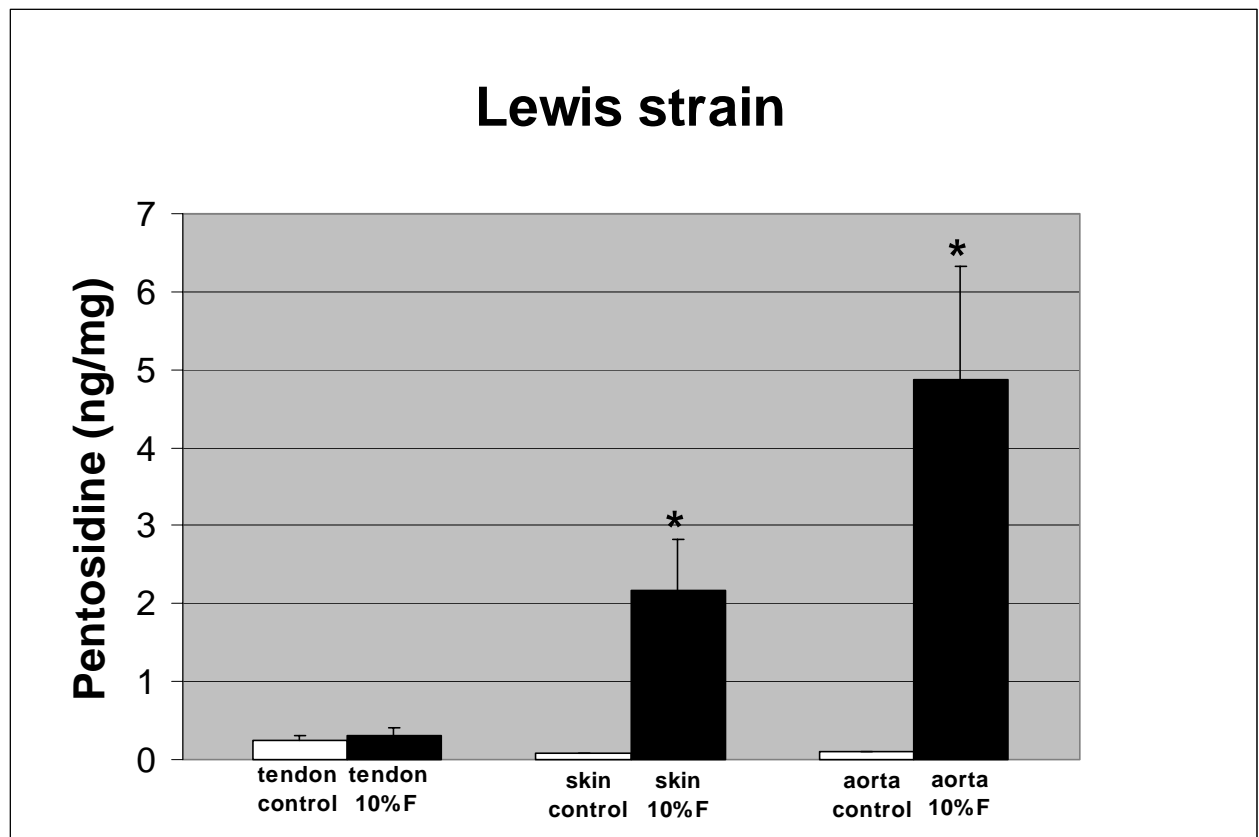


Fig. 3

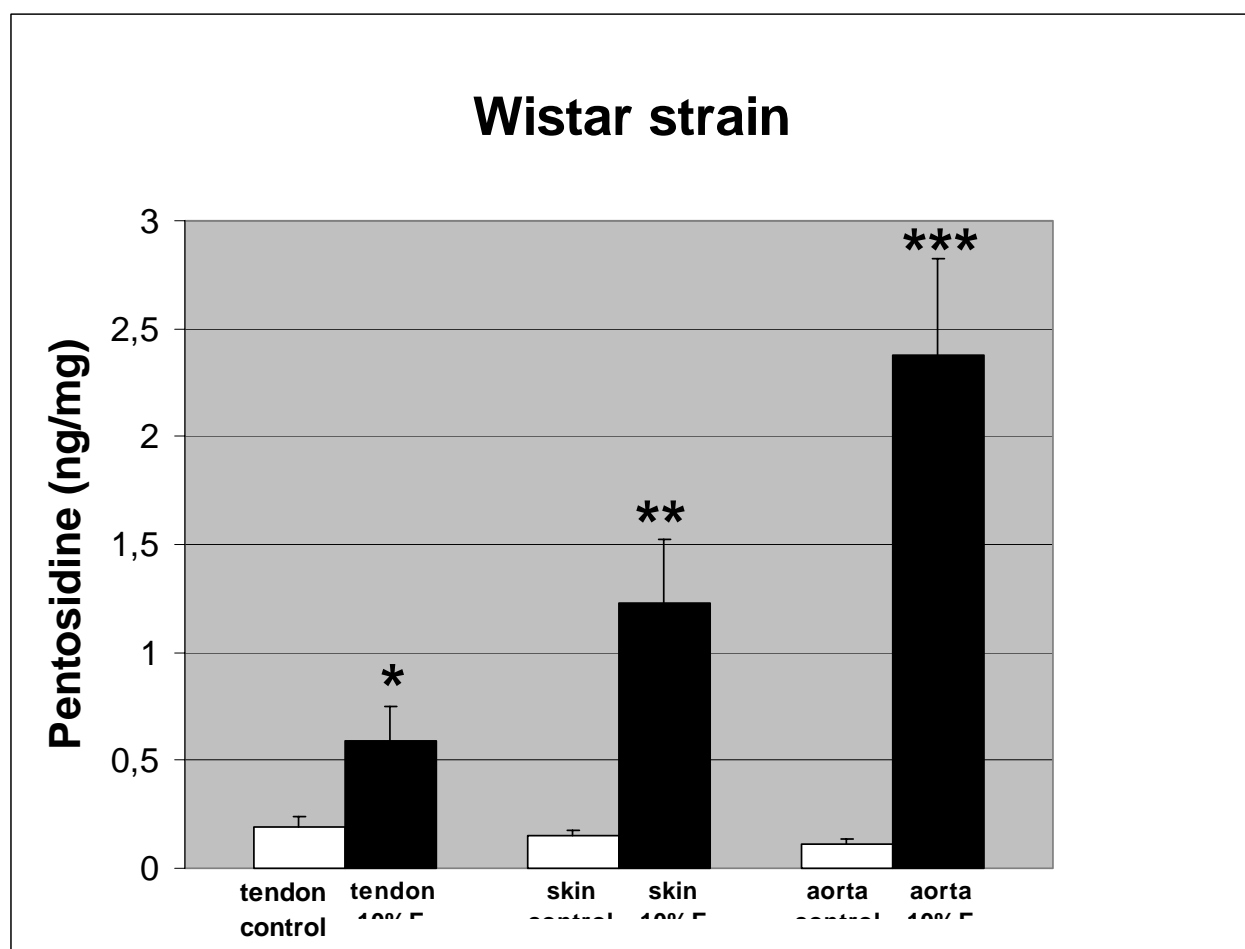


Fig. 4

