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Age- and feeding-dependent production of carbonyl compounds in hypoxic heart. The role of carbonyls produced in connective tissue modification

Zdeněk Deyl*, Ivan Mikšík

Institute of Physiology Academy of Sciences of the Czech Republic Vídeňska 1083 142 20 Prague 4 Czech Republic

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Abstract

The concentration of reactive lipid metabolites (malondialdehyde, formaldehyde, acetaldehyde and acetone) was assayed in rat heart reperfusates after 30 min ischemia in animals of different age and kept on different feeding regimes It was revealed that there is no difference in the concentration of reactive carbonyl compounds in reperfusates from animals of different age, but the amount of released carbonyl compounds is much lower in animals kept on 50% restricted diet If tail tendons from young (3 months) rats are incubated in the reperfusate, their solubility after CNBr treatment is decreased so that this material resembles tendons from old animals Also the amino acid composition of the insoluble residue cannot be distinguished from that obtained from rat tail tendons of 24- or 29-month-old rats The results prove the ability of carbonyl containing lipid metabolites to create a CNBr-insoluble core in connective tissue

Key words Non-enzymatic glycation, Food restriction, Carbonyls, Collagen, Aging, Hypoxic heart

1. Introduction

Carbonyl compounds, in particular malondialdehyde, formaldehyde, acetaldehyde and acetone represent reactive metabolites (products of peroxidation) of un-

* Corresponding author

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saturated lipids The compounds are considerably reactive and their involvment in non-enzymatic modification of proteins has been the subject of several studies [1,2] From their chemical nature they are likely to compete for similar reactive sites in biopolymers as aldehydic sugars, which are known to produce advanced glycosylation end products (AGEs) by reactions known as non-enzymatic glycation or Maillard reaction [3–6]

Moreover, glycosylaminoacids and their Amadori rearrangement products could co-exist and react with malondialdehyde and the highly reactive intermediates derived therefrom A thorough in vitro study on the effect of malondialdehyde on the Maillard degradation of Amadori compounds was published by Gomez-Sanchez et al [7]

In previous studies [8,9] we investigated changes occurring in connective tissue in fully fed and food restricted animals with age, in particular we followed changes in UV absorbance, fluorescence, thiobarbituric test response and hexosyllysine It turned out that animals which experienced restriction for a long-enough period of time survived better and were lower in all the above parameters This indicated a lower concentration of AGEs or their intermediates Masoro et al [10] described that young food restricted rats exhibit a decreased percentage of glycosylated hemoglobin (about 30–40%) when compared with ad libitum fed counterparts and this difference disappeared as the animals grew older (Masoro, as quoted by Monnier [5]) In contrast, a decrease in glycemia of 15–20% persisted in the food restricted animals It seemed therefore reasonable to speculate on whether or not other sources of carbonyls may be present in tissues that may lead products similar to AGEs or which may compete for the glucose binding sites in proteins Obviously, lipid peroxidation products appeared to be candidates for this category of reactions In this study we explored the Langendorff heart perfusion in animals of different age and kept on different regimes The perfusates we retested for the presence and amount of lipid peroxidation products and their effect upon connective tissue

2. Material and methods

2 1 Animals

Four groups of male Wistar rats (10 animals per group) were used The animals were kept separately (a single animal per cage), fed standard pelletted diet with a free access to water The feeding regime in individual groups ran as follows Group 1 First year, free access to food, second year, free access to food Group 2 First year, 50% food restriction, second year 50% food restriction Group 3 First year, free access to food, second year, 50% food restriction Group 4 First year, 50% food restriction, second year, free access to food

The 50% food restriction refers to the amount of food consumed by unrestricted controls and was adjusted weekly With fully fed animals (Group 1) and food restricted animals (Group 2) experiments were done after one year of the feeding regime as well

Body weight changes during the growth of experimental animals were not significantly different from those previously reported [11]

2 2 Isolated heart preparation

The procedure used followed that published by Otani et al [12] The rats were anesthesized by intraperitoneal injection of pentobarbital (80 mg/kg body mass) and hearts were removed and mounted on a non-recirculating Langendorff perfusion apparatus as rapidly as possible Retrograde perfusion was done at pressure of 100 cmH_2O ($9\,8 \times 10^3$ Pa) with oxygenated, normothermic Krebs-Henseleit bicarbonate buffer containing 3% serum albumin First the heart preparation was allowed to equilibrate for 10 min at 37°C with nonrecirculating Krebs-Henseleit bicarbonate buffer The retrograde aortic flow was then terminated and the hearts were made ischaemic for 30 min in physiological saline at 37°C Then reperfusion was started with the Krebs-Henseleit solution for 60 min at normothermia Samples of the perfusate (two perfusion times, namely 15 and 45 min) were collected prior to ischemia, after ischemia and during reperfusion and assayed for lipid metabolic products

2 3 Derivatization of metabolites in perfusate

This procedure followed that of Cordis et al [13] Samples taken during the individual stages of perfusion were derivatized with 2,4-dinitrophenylhydrazine (DNPH) DNPH (310 mg) was dissolved in 100 ml of 0 2 M HCl and 0 1 ml of this DNPH reagent (3 13 μmol) was added to 1 5 ml of the perfusate in a 20-ml screw-capped PTFE vial After adding 0 5 ml of water the test tubes were Vortex mixed, after mixing 10 ml of *n*-pentane were added The test tubes were shaken for 30 min and the derivatization reaction was allowed to proceed at room temperature The organic phase was removed and the aqueous phase was reextracted with the same amount of pentane, both extracts were combined and taken to dryness under a stream of nitrogen at 30°C The dry residue was redissolved in 200 μl of acetonitrile and after filtration directly used (0 2 μm Nylon-66 membrane filter, Rainin, Woburn, MA) for chromatography

2 4 Chromatography of 2,4-dinitrophenylhydrazones [10]

The chromatographic procedure followed that described by Cordis et al [13] Filtered samples (25 μl) were loaded in a 3-m Beckman Ultrasphere C_{18} column 7 5 cm × 4 6 mm i d in a Spectra-Physics Chromatograph (San Jose, CA) Injection of samples was done by the split stream technique, a Bondapak C_{18} Guard Pak column was mounted into the separation system Detection was by a Waters model 490 multiwavelength detector (Millipore, Milford, MA) at 307, 325 and 356 mm Elution was isocratic with acetonitrile-water-acetic acid (40 60 0 1, v/v/v) at a flow rate of 1 ml/min (typical running time less than 20 min) After each run the column was washed with acetonitrile containing 0 1% (v/v) of acetic acid

Dinitrophenylhydrazone standards were prepared in the laboratory by reacting 50 ml 2,4-dinitrophenylhydrazine solution (310 mg/100 ml 2 M HCl) with a 2–5 molar excess of formaldehyde (FDA), acetaldehyde (ADA), malondialdehyde (MDA) and acetone The hydrazone precipitate was filtered off, dried and recrystallized from methanol Standard solutions of 2,4-dinitrophenylhydrazones containing 50 ng/μl were used for spiking Calibration was linear in the range from 10 pmol to 6 25 nmol of each standard Within-run and inter-run variations were 1 and 5%, respectively

2 5 In vitro reaction of tail tendons with lipid metabolites

Intact rat tendon fibres were incubated with the 45-min perfusate in vitro Fibres were washed overnight at 10°C in 5 mM sodium phosphate buffer containing 0 9% NaCl, pH 7 4 and then incubated with the 45-min perfusate which was made 3 mM with respect to sodium azide at 37°C for 24 h

2 6 Amino acid analysis

Amino acid analysis was done on PICO-TAG Amino Acid Analysis System with standard derivatization with phenyl isothiocyanate Briefly, protein was hydrolyzed in 6 M HCl overnight at 110°C, derivatized by phenyl isothiocyanate to the phenylthiocarbamyl amino acids and these amino acids were separated by reversed phase high performance liquid chromatography with sodium acetate buffer (pH 6 40) — acetonitrile gradient monitored at 254 nm (for details see Bidlingmeyer et al [14])

3. Results

As demonstrated in Table 1, after 30 min ischemia, heart perfusates contain lipid metabolites, reactive carbonyl compounds, namely MDA, FDA, ADA and acetone While there are no significant differences between perfusates obtained from animals aged 1 and 2 years (fully fed and food restricted animals), the feeding regime changes the concentration of reactive carbonyls quite considerably When fully fed animals are compared with the 50% food restricted ones, the concentration of reactive carbonyls in the perfusate drops by nearly 50% in the group of food restricted animals It appears that the concentration of the reactive carbonyls in heart perfusate (both 45- and 15-min perfusates) reflects the actual feeding regime rather than the nutritional history thus the results obtained with 2-year-old animals which were fully fed during the first year and food restricted during the second year of their life resembles the situation in animals food restricted for the whole testing period Vice versa, animals food restricted in their early life and fully fed then after yielded perfusates in which the concentrations of reactive carbonyl metabolites resembled fully fed animals

In Table 2 the differences in the percentage of CNBr insoluble residue with age and dietary regime and the effect of incubation with a mixture of lipid metabolites are shown In samples which were not incubated with the mixture of lipid metabolites, the proportion of the insoluble residue was lower in all groups tested (when compared with the incubated ones) There was a statistically significant increase in the insoluble residue in fully fed animals after their first and second year of life The analogous difference in food restricted animals was considerably less pronounced

Comparison of the incubated and non-incubated samples shows a clear increase in the CNBr insoluble residue if the samples are incubated with the mixture of lipid metabolites (see above) The decrease in solubility with age in fully fed and food restricted animals after the first and second year of life is even more pronounced in incubated samples than in the non-incubated ones As a matter of fact the resistence towards solubilization of incubated samples is significantly higher than the most resistant non-incubated samples obtained from 2-year-old fully fed animals This in-

Table 1
Concentrations of lipid metabolites in rat heart perfusates after 30 min ischemia with reperfusion of 15 and 45 min (mean ± S D , $N = 5$)

Regime	Age	MDA-DNPH	FDA-DNPH	ADA-DNPH	Acetone-DNPH
Perfusion time 15 min					
Fully fed	1 year	0 08 ± 0 02	3 15 ± 1 22	3 98 ± 1 89	20 01 ± 2 56
Fully fed	2 years	0 09 ± 0 02	3 27 ± 0 83	4 11 ± 1 62	21 02 ± 3 56
Food restricted	1 year	0 05 ± 0 02[a]	1 52 ± 0 63[a]	1 26 ± 0 83[b]	12 52 ± 2 55[c]
Food restricted	2 years	0 04 ± 0 01[c]	1 36 ± 0 32[c]	1 34 ± 0 79[c]	11 85 ± 1 96[d]
Fully fed, restricted	2 years	0 04 ± 0 01[c]	1 40 ± 0 32[c]	1 38 ± 0 81[c]	14 22 ± 2 02[c]
Food restricted, fully fed	2 years	0 08 ± 0 02[g,j]	3 22 ± 0 96[g,j]	3 99 ± 2 02[e,i]	20 22 ± 2 15[h,j]
Perfusion time 45 min					
Fully fed	1 year	0 15 ± 0 04	5 98 ± 2 03	5 63 ± 1 46	20 40 ± 1 98
Fully fed	2 years	0 18 ± 0 06	5 03 ± 2 11	5 66 ± 1 62	21 40 ± 2 03
Food restricted	1 year	0 06 ± 0 03[c]	2 65 ± 0 84[c]	2 73 ± 0 57[c]	14 23 ± 1 26[d]
Food restricted	2 years	0 05 ± 0 03[c]	2 49 ± 0 92[a]	2 84 ± 0 62[c]	13 21 ± 1 22[d]
Fully fed restricted	2 years	0 07 ± 0 04[c]	3 02 ± 1 06	3 26 ± 0 89[b]	15 21 ± 2 03[c]
Food restricted, fully fed	2 years	0 14 ± 0 05[g,i]	5 93 ± 2 26[f,i]	5 78 ± 1 98[f,i]	19 22 ± 1 56[h,j]

Metabolites malondaldehyde, MDA, formaldehyde FDA acetaldehyde ADA, acetone
[a] $P < 0\ 05$, comparison with fully fed [b] $P < 0\ 02$, comparison with fully fed [c] $P < 0\ 01$ comparison with fully fed [d] $P < 0\ 001$, comparison with fully fed [e] $P < 0\ 05$, comparison with food restricted [f] $P < 0\ 02$, comparison with food restricted [g] $P < 0\ 01$, comparison with food restricted [h] $P < 0\ 001$ comparison with food restricted [i] $P < 0\ 05$, comparison with fully fed and then restricted [j] $P < 0\ 01$ comparison with fully fed and then restricted

Table 2
Changes in the percentage of CNBr insoluble residue with age and dietary regime and the effect of incubation with a mixture of lipid metabolites (mean ± S.D. $N = 10$)

Regime	Age	Insoluble residue (weight %)	
		Non incubated	Incubated
Fully fed	1 year	11.36 ± 2.17	18.24 ± 1.12
Fully fed	2 years	14.28 ± 2.15[c]	23.05 ± 1.08[c]
Food restricted	1 year	5.48 ± 1.23[b]	18.26 ± 0.96
Food restricted	2 years	7.21 ± 2.15[b,d]	24.15 ± 1.24[b,c]
Fully fed, restricted	2 years	6.25 ± 2.22[b]	19.37 ± 2.05[b,c]
Food restricted fully fed	2 years	6.24 ± 3.11[b]	19.96 ± 1.18[b,c]

Tendons obtained from animals of the groups as indicated. 45 min perfusate from fully fed animals used for incubation.
$P < 0.001$ difference between incubated and non-incubated counterparts is always significant. [a] $P < 0.05$, comparison with fully fed. [b] $P < 0.001$ comparison with fully fed. [c] $P < 0.001$ comparison with food restricted. [d] $P < 0.05$ comparison between age categories (1 and 2 years). [e] $P < 0.001$ comparison between age categories (1 and 2 years).

dicates that under physiological conditions the tissue though more polymerized with advancing age is susceptible to further polymerization through lipid oxidation products. Samples obtained from animals subjected to alternating feeding regimes (fully fed first year and undernourished during the second year and vice versa) are polymerized to a lower degree than the samples obtained from fully fed or undernourished animals.

If rat tail tendons obtained from 3-month-old rats (which are 92.2 ± 2.3% soluble after CNBr treatment) were used, they yielded 16.24 ± 1.36% ($n = 10$) of the insoluble residue.

Amino acid analyses of the CNBr insoluble residues (Table 3) indicate that except the residues obtained from 3-month-old animals there is very little change in their composition. It should be noted that apparently the CNBr cleavage was incomplete as always some methionine residues persisted in the analysed insoluble residues. The CNBr resistant portion of the tendon obtained from 3-month-old animals is considerably higher in its content of proline and lower in glycine compared to all the other samples analyzed. These residues are likely to be composed of collagen and the accompanying non-collagenous proteins, the proportions of which may change with age. Incubation of rat tail tendons obtained from 3-month-old animals in the 45-min perfusate containing the reactive carbonyls not only increases the amount of the CNBr inaccessible residue as mentioned above, but also its composition was changed in a way that it resembled the resistant material obtained from 24- or 29-month-old rats.

4 Discussion

Reactive carbonyl metabolites, particularly those containing a free aldehydic group are known to react with free amino groups of proteins, such reactions are best

Table 3
Amino acid composition of the CNBr insoluble residues obtained from rats of different age and remaining after cleavage of tail tendons obtained from 3 months old rats incubated in the 45 min perfusate (fully fed animals)

Amino acid	Animal age (months)				
	3	12	24	29	3′
Asp	2 67	3 21	3 11	3 17	3 19
Glu	3 41	4 27	5 00	5 30	5 33
Hypro	15 16	15 02	14 03	14 28	14 18
Ser	3 40	3 54	3 41	3 41	3 42
Gly	18 12	22 79	23 51	23 49	23 62
Arg	2 21	3 26	3 46	3 45	3 43
Thr	2 76	2 73	2 80	2 88	2 83
Ala	4 05	5 33	5 96	6 32	6 03
Pro	35 04	26 04	24 11	24 58	24 53
Tyr	1 35	1 32	1 18	1 27	1 28
Val	2 21	2 46	2 57	2 46	2 50
Met	0 28	0 33	0 57	0 54	0 57
Cys	1 11	0 87	0 30	0 23	0 23
Ileu	1 99	1 78	2 36	1 91	2 03
Leu	2 44	3 26	3 80	3 45	3 53
Phe	3 09	3 09	2 96	2 28	2 32
Lys	0 72	0 76	0 99	0 98	0 99

For details of the incubation procedure see Material and methods
3′, incubated in 45-min perfusate

detected in protein structures with a long metabolic half-time A typical representative of this protein category is collagen (for a review of glycation of collagen see Ref 15) However, instead of using purified collagen samples to test the ability of carbonyl containing perfusates to react with the protein, we have used rat tail tendons, these tendons are known to contain mainly collagen (type I), however they exhibit a definite supramolecular structure and experiments carried out with them are closer to the physiological conditions in the organism The presumptive counterparts for the reactive carbonyl containing metabolites, free amino groups, are much more readily accessible in a solubilized protein in comparison to a protein imbedded in a tissue Application of such a model situation has, however, to respect that a part of the protein in the tissue (rat tail tendon) may be already reacted (cross-linked) with reactive metabolites or otherwise Therefore it was necessary to use a standard method for solubilizing tendon collagen This was done by CNBr cleavage which is known to split collagen type I molecule into six fragments, collagen molecules which underwent a reaction with reactive carbonyls (including aldehydic sugars) are known to form polymeric structures, parts of which remain insoluble even after intensive CNBr cleavage On the other hand it has been proposed that aging of connective tissue involves collagen polymerization [16,17] The nature of some of the cross-links arising is known, however the complete mechanism of age-accompanied collagen

polymerization has been a matter of debate for the last 20 years Indeed if we compare the percentage of tendons that remain insoluble after CNBr cleavage some increased resistance towards solubilization with CNBr can be observed (Table 2) The same tendency was observed by Tanaka et al [18] in the case of incubation of rat tail tendom with ribose

It can be concluded, that the reactive carbonyls present in tissue, e g after an ischaemic period, are capable of polymerizing tissue collagen

5. Conclusions

Reperfusion of rat heart after 30 min ischemia carried out during a 15- or 45-min period yields perfusates containing reactive metabolites with carbonyl groups The concentration of these metabolites depends on the nutritional regime and is about twice as high in fully fed animals as compared to those that were 50% restricted in their food intake Previous feeding history seems not to affect the concentration of released metabolites Also no distinct difference in the concentration of the carbonyl containing compounds were found between 1- and 2-year-old animals

The released carbonyl bearing metabolites are able to react with connective tissue (rat tail tendons), increasing its resistance to CNBr cleavage This indicates that they should take part in polymerization reactions of the constitutive proteins The amino acid composition of the solubilization-resistant core resembles the composition of the insoluble residues (after CNBr cleavage) obtained from tail tendons of older 12- and 24-months-old rats It is worth mentioning that this composition differs from that occurring physiologically in young 3-month-old rat tail tendons In our opinion the presented results are evidence of the ability of reactive lipid metabolites to react (polymerize) with connective tissue structures The modified insoluble connective tissue, as far as its amino acid composition and proportion of the insoluble residue is concerned, is indistinguishible from insoluble connective tissue fraction obtained from old animals

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