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Influence of curcumin treatment on collagen metabolism in experimental myocardial necrosis in rats

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Abstract. This study was carried out to evaluate whether curcumin, a potent antioxidant, had any specific role in the synthesis and degradation of collagen in rat heart with myocardial necrosis, induced by isoproterenol.HCl (ISO). Myocardial necrosis was induced by administration of ISO (30 mg/100 g body weight subcutaneously twice at an interval of 24 h) and studies on collagen metabolism were carried out with curcumin (200 mg/kg) pre- and co-treatment with ISO. The incorporation of ^{14}C-proline into collagen was studied as an index of collagen synthesis. Increased fractional synthesis rate and enhanced degradation of newly synthesized collagen were observed in ISO administered animals. Curcumin pre- and co-treatment with ISO was noticed to decrease the degree of degradation of the existing collagen matrix and collagen synthesis, two weeks after the second dose of ISO. The observed effects could be due to free radical scavenging capacity and inhibition of lysosomal enzyme release by curcumin.

Keywords. Curcumin; myocardial necrosis; isoproterenol; collagen; free radical; lysosomal enzymes.

1. Introduction

Collagen is an integral part of the normal extracellular matrix of the heart, linking the myriad myocardial tissue components into a cohesive whole and ensuring efficient function as a biological pump [1]. Of the non-muscle cells present in the heart, over 90% are fibroblasts, which are involved in collagen synthesis [2]. Increased cardiac collagen synthesis and degradation of existing collagen have been observed in experimental myocardial necrosis and altered cardiac function [3–5]. Increased awareness of the importance of myocardial collagen in health and disease has led to the search for drugs which could protect the existing collagen matrix and enhance the healing process.

Curcumin (1,7-*bis*(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a major component of turmeric.Turmeric is used as a spice and food colourant and has been used for its various medicinal properties in folk medicine [6]. Curcumin exhibits antioxidant [7] as well as antiinflammatory activity [8], inhibits cancer growth [9] and HIV replication [10]. More recently, curcumin is shown to have antiviral activity [11]. We have observed earlier that curcumin inhibits the release of lysosomal enzymes thereby enhancing the stability of lysosomal membranes during myocardial necrosis [12].

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The objective of this study is to evaluate whether curcumin could influence the *in vivo* rates of collagen turnover during ISO induced myocardial necrosis in rats. The collagen metabolism is investigated *in vivo*, by administering radiolabelled proline with a "flooding" dose of unlabelled proline [13] and this method allows simultaneous measurement of the rates of collagen synthesis as well as degradation.

2. Materials and methods

Isoproterenol.HCl was purchased from Sigma Chemical Co., USA. ^{14}C-Proline was obtained from Bhabha Atomic Research Centre, Mumbai. Diphenylamine and orcinol were purchased from SD's Fine Chemicals, Mumbai, India. All other chemicals used were of analytical grade.

Female rats (Wistar, inbred at the Central Leather Research Institute, Chennai, animal facility) weighing approximately 100 g, were housed in solid-bottomed polypropylene cages. The animals received commercial rat diet and water *ad libitum*. The animals were divided into four groups as follows; (1) Group 1 – Normal control; (2) Group 2 – ISO administered (3) Group 3 – Curcumin treated control; (4) Group 4 – Curcumin pre- and co-treated with ISO. Curcumin (200 mg/kg body weight) suspended in 1% gum acacia in water was given to each animal orally for two days prior to ISO administration and the same dose was continued during ISO administration. After the experimental period (one day, one week and two weeks after the second dose of ISO), the rats were sacrificed by cervical decapitation.

3. Collagen metabolism studies

Three hours before sacrifice, animals were injected with ^{14}C-proline (40 μci/100 g body weight) with a flooding dose of unlabelled proline (1·4 mmol/100 g body weight) in saline (0·5 ml) intraperitoneally. After sacrifice, the heart was perfused with cold saline, weighed, minced, homogenized in ice cold water and mixed with equal volume of 10% trichloroacetic acid (TCA). The homogenate was centrifuged for 15 min at 15,000 × g. The TCA soluble fraction was removed, neutralised with 1 N NaOH and this formed the tissue free pool. The TCA insoluble pellet was washed twice with cold 5% TCA, followed by ethanol : ether (1:1) and finally with ether. The pellet was dried, weighed and hydrolysed in 6 N HCl at 110°C for 18 h.

The proline and hydroxyproline contents, total radioactivity and specific radioactivity of proline and hydroxyproline were measured in total proteinaceous and in tissue free pool (hydrolysed and unhydrolysed supernatants). This was achieved by oxidizing samples with chloramine T and extracting the products into toluene as described by Rojkind and Gonzalez [14].

Fractional synthesis rates (Ks) for collagen were calculated as described below [15] using the formula

$$\text{Ks (\% day)} = \frac{\text{tissue } ^{14}\text{C-hydroxyproline specific radioactivity}}{^{14}\text{C-proline specific activity in tissue free pool} \times \text{time following injection}} \times 100$$

The specific radioactivity of tissue ^{14}C-hydroxyproline was derived from radioactivity in both protein and tissue free pool.

Degradation of newly synthesized collagen (%) was estimated as described previously [16] *viz.*·

$$\frac{\text{Radioactive hydroxyproline in tissue free pool (cpm)}}{\text{Total radioactive hydroxyproline in tissue (cpm)}} \times 100 .$$

An index of the breakdown of "mature" collagen in the extracellular matrix was obtained from the ratio of molar amounts of free hydroxyproline to the total amount of hydroxyproline in the tissue [16].

4. Statistical analysis

The results were statistically evaluated using one way analysis of variance (ANOVA) for repeated measurements. When F values indicated significant differences, they were further evaluated with calculation of least significant difference (LSD) to check whether the mean differences were significant. Multiple group difference analysis for all parameters have been evaluated.

5. Results and disussion

Table 1 shows the effect of curcumin on urine, serum and heart hydroxyproline levels. Urine and serum hydroxyproline levels were found to be significantly increased while a

Table 1. Effect of curcumin on urine, serum and heart hydroxyproline in ISO-induced myocardial necrosis.

Group	Urine hydroxyproline μg/mg creatinine	Serum hydroxyproline μg/ml	Heart hydroxyproline mg/g dry tissue
A			
Group 1	160 ± 1·81	42·80 ± 0·65	5·51 ± 0·106
Group 2	278 ± 1·19**	68·16 ± 1·08**	4·41 ± 0·079**
Group 3	166 ± 2·43	44·17 ± 1·49	5·39 ± 0·096
Group 4	170 ± 2·17*[a]	50.83 ± 0.91*[a]	5.03 ± 0.100*[b]
B			
Group 1	164 ± 3·84	40·70 ± 1·81	6·21 ± 0·15
Group 2	235 ± 3·43**	54·31 ± 1·15**	4·46 ± 0·19**
Group 3	164 ± 3·13	38·24 ± 0·90	6·36 ± 0·13
Group 4	191 ± 5·28**	45·46 ± 0·89*[a]	5·35 ± 0·13**
C			
Group 1	173 ± 3·97	43·42 ± 0·91	6·38 ± 0·12
Group 2	200 ± 4·58**	49·37 ± 1·52**	5·03 ± 0·20**
Group 3	173 ± 4·33	40·81 ± 1·52	6·32 ± 0·15
Group 4	177 ± 4·11[b]	43·06 ± 1·05[a]	6·21 ± 0·15[a]

A – one day after the second dose of Isoproterenol; *B* – one week after the second dose of ISO; *C* – two weeks after the second dose of ISO

Group 1 – Normal control; Group 2 – ISO administered ; Group 3 – curcumin treated control; Group 4 – curcumin pre- and co-treated with ISO

Values are mean ± SE of six samples

**$P < 0.001$, *$P < 0.01$, as compared to control; [a]$P < 0.001$; [b]$P < 0.01$, as compared to ISO

significant decrease in heart hydroxyproline was observed in Group 2 when compared to Group 1, one day after the second dose of ISO. One week after the second dose of ISO in Group 4, the serum, urine and heart hydroxyproline levels were found to be statistically significant when compared to Group 1 and 2, but they were comparable to Group 1 two weeks after the second dose of ISO.

Tissue hydroxyproline, total and specific activities of hydroxyproline are shown in table 2. Significantly decreased levels of hydroxyproline and increased total and specific activities were observed in Group 2 when compared to Group 1, one day after the second dose of ISO. Tissue hydroxyproline level in Group 4 was found statistically significant when compared to Group 1 and Group 2.

One week after the second dose of ISO, the tissue hydroxyproline level and the total and specific activities of hydroxyproline in Group 2 were found to be significant when compared to Group 1 and the values observed in Group 4 were significant when compared to Group 1 and 2. Two weeks after the second dose of ISO, the heart hydroxyproline level and total and specific activities of hydroxyproline observed in Group 4 were significant when compared to Group 1 and 2.

The data on collagen synthesis and degradation are presented in table 3. Fractional collagen synthesis rate was significantly increased one day after the second dose of ISO in Group 2 when compared to Group 1 and in Group 4 also, level was significant when compared to Group 1 and it was found to be significantly decreased when compared to Group 2. There was a significant increase in the degradation of newly synthesized

Table 2. Effect of curcumin on total hydroxyproline, and on total and specific activities of ^{14}C-hydroxyproline in ISO induced myocardial necrosis.

Group	Hydroxyproline μmoles/g wet tissue	Total activity cpm/g wet tissue	Specific activity cpm/μmoles
A			
Group 1	6·12 ± 0·28	1174 ± 61	226 ± 12
Group 2	4·69 ± 0·25**	1799 ± 41**	324 ± 15**
Group 3	6·75 ± 0·30	1328 ± 98	234 ± 15
Group 4	5·58 ± 0·21^{a}	1524 ± 58*^{b}	291 ± 17*^{b}
B			
Group 1	5·81 ± 0·20	1644 ± 44	95 ± 3·3
Group 2	4·14 ± 0·23**	981 ± 28**	276 ± 9·3**
Group 3	3·07 ± 0·19**	1700 ± 34	95 ± 4·1
Group 4	9·48 ± 0·30**^{a}	2229 ± 67**^{a}	219 ± 7·4**^{a}
C			
Group 1	5·34 ± 0·16	1645 ± 32	93 ± 2·5
Group 2	2·48 ± 0·11**	1592 ± 31	200 ± 7·5**
Group 3	3·30 ± 0·08**	1628 ± 52	88 ± 2·8
Group 4	6·13 ± 0·25*^{a}	731 ± 36**^{a}	124 ± 5·3**^{a}

A – one day after the second dose of ISO; B – one week after the second dose of ISO; C – two weeks after the second dose of ISO

Group 1 – normal control; Group 2 – ISO administered; Group 3 – curcumin treated control; Group 4 – curcumin pre- and co-treated with ISO

Values are mean ± SE of six samples

**$P < 0·001$, *$P < 0·01$, as compared to control, $^{a}P < 0·001$; $^{b}P < 0·01$, as compared to ISO

Table 3. Influence of curcumin on synthesis and degradation of collagen in ISO induced myocardial necrosis.

Group	Fractional synthesis rate % day	Degradation of newly synthesized collagen (%)	Maturation collagen degraded (%)
A			
Group 1	7·96 ± 0·24	55·98 ± 2·19	1·48 ± 0·05
Group 2	19·41 ± 0·50**	70·54 ± 5·40**	4·43 ± 0·06**
Group 3	8·43 ± 0·33	57·25 ± 1·18	1·49 ± 0·04
Group 4	10·31 ± 0·46**[a]	61·55 ± 1·30*[a]	2·20 ± 0·03*[a]
B			
Group 1	7·72 ± 0·25	53·44 ± 1·83	2·90 ± 0·19
Group 2	16·41 ± 0·62**	42·95 ± 1·75**	1·79 ± 0·14**
Group 3	8·27 ± 0·15	53·89 ± 1·16	2·84 ± 0·13
Group 4	13·07 ± 0·32**[a]	41·78 ± 1·05**	2·36 ± 0·14[b]
C			
Group 1	8·89 ± 0·22	52·33 ± 1·50	3·12 ± 0·16
Group 2	13·06 ± 0·58**	46·68 ± 2·00	1·47 ± 0·09**
Group 3	8·88 ± 0·19	51·97 ± 2·31	3·13 ± 0·19
Group 4	10·14 ± 0·35**[a]	40·18 ± 1·56**[a]	2·36 ± 0·10**[a]

A – one day after the second dose of ISO; B – one week after the second dose of ISO; C – two weeks after the second dose of ISO

Group 1 – normal control; Group 2 – ISO administered; Group 3 – curcumin treated control; Group 4 – curcumin pre- and co-treated with ISO

Values are mean ± SE of six samples

$^{**}P < 0.001$, $^{*}P < 0.01$, as compared to control, $^{a}P < 0.001$; $^{b}P < 0.01$, as compared to ISO

collagen in Group 2 when compared to Group 1 while curcumin treatment (Group 4) decreased the degradation but the level was still significant when compared to Group 1 and 2. The degradation of matured collagen was increased in Group 2 but it was comparatively less in Group 4, however it was found to be significant when compared to Group 1 and 2. Two weeks after the second dose of ISO, the values for fractional synthesis, degradation of newly synthesized collagen and mature collagen degradation (Group 4) were observed to be significant when compared to Group 1 and 2.

Administration of ^{14}C-proline and subsequent measurement of ^{14}C-hydroxyproline in heart as well as hydroxyproline in serum and heart provide valuable information on the metabolic turnover of collagen, its synthesis and degradation. Myocardial hypertrophy in rats appears one day after ISO administration and this might be due to the increased water content, edematous intermuscular space and extensive necrosis of cardiac muscle followed by invasion of the damaged tissue by inflammatory cells [17,18]. Hypertrophy has been characterized as a compensatory response to myocyte loss [19].

The correlation between collagen concentration and intracellular degradation is of interest and it may have a role in the regulation of collagen content. Schneir *et al* [20] have suggested an increased degradation of newly synthesized collagen during streptozotocin induced diabetes but during experimentally induced pulmonary fibrosis, a decreased degradation of newly synthesized collagen might contribute to the collagen deposition in the early stages [21]. The increased rate of newly synthesized collagen degradation observed

in isoproterenol group could be due to multiple reasons. The proportion of newly synthesized collagen degradation is influenced by the intracellular concentration of cAMP[22]. In an earlier study, an increase in the cAMP levels is observed after isoproterenol injection, with resultant increase in calcium[23]. cAMP might activate a specific intracellular protease or it might activate a protein kinase capable of tagging collagen molecules for degradation[24]. Inhibition of lysosomal enzymes suppresses the degradation, suggesting that lysosomes may be the sites of action for this process[25]. Our earlier report[12] shows that curcumin inhibits the release of lysosomal enzymes and this could be the cause for the decreased degradation of newly synthesized collagen in this study.

Eeckhout and Vaes[26] have presented evidence showing that the collagenase and its endogenous activator are present in latent form which could be activated by lysosomal proteinases. Curcumin, by lowering the levels of lysosomal hydrolases could contribute to the decreased activity of collagenase resulting in a decrease in mature collagen degradation. The observation of curcumin treatment decreasing the urine and serum hydroxyproline levels, two weeks after the second dose of ISO in this study, also supports the fact that curcumin partially protects the existing collagen.

Molecular oxygen is involved in the hydroxylation of proline to hydroxyproline and prolyl hydroxylase activity. Free radical generation is involved in the process of isoproterenol induced cardiac damage[27]. The observation of increase in fractional synthesis rate in isoproterenol administered group in this study shows that prolyl hydroxylase may be activated during myocardial necrosis. It has been reported earlier that the activity of prolyl hydroxylase is elevated in experimental myocardial infarction[28]. The fact that molecules which inhibit lipid peroxidation also inhibit collagen synthesis suggests that there is an interrelationship between the two phenomena. Our earlier study also demonstrates the inhibition of lipid peroxidation by curcumin during myocardial necrosis[17]. *In vitro* experiments show that the inhibition of collagen synthesis by retinoids could be due to their antioxidant activity[29]. The inhibition of collagen synthesis by curcumin observed in this study, could also be due to its antioxidant activity[17].

In conclusion, the results demonstrate that curcumin partially prevents the degradation of the existing collagen matrix and helps to decrease the collagen synthesis.

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