PREVENTION OF ISOPROTERENOL-INDUCED CARDIAC HYPERTROPHY BY EUGENOL, AN ANTIOXIDANT

Rashmi Choudhary¹, K. P. Mishra² and C. Subramanyam¹

¹Department of Biochemistry, Osmania University, Hyderabad 500 007 and

²Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400 085, INDIA

ABSTRACT

Recent reports on the involvement of calcineurin in cardiac hypertrophy and its susceptibility to free radicals, prompted us to examine possible beneficial effects of dietary antioxidants in this regard. In continuation of initial *in vitro* studies revealing eugenol to be a potent calcineurin inhibitor, we investigated its ability to reverse isoproterenol-induced cardiac hypertrophy in rats. Intraperitoneal administration of isoproterenol (1 mg/kg body wt/day for 10 days) induced cardiac hypertrophy with increased heart weight and enhanced apoptosis of myocytes concomitant with accumulation of reactive oxygen species, decreased glutathione contents, increased activities of calcineurin and protein kinase C in ventricular tissue. Administering eugenol for 3 days (1 mg/kg body wt/ twice a day), followed by combined administration of isoproterenol and eugenol resulted in significant reversal of cardiac hypertrophy and restoration of above changes. These results suggest that eugenol, a natural antioxidant of dietary origin, may offer potential benefits in the management of cardiac hypertrophy.

KEY WORDS

Antioxidants, Calcineurin activity, Cardiac hypertrophy, Eugenol, ROS, Isoproterenol.

INTRODUCTION

Cardiac hypertrophy is an adaptive response of the heart muscle to a wide variety of intrinsic and extrinsic stimuli. Although hypertrophy of the heart muscle is initially beneficial during early growth, prolonged hypertrophy is potentially deleterious causing dilated cardiomyopathy and heart failure causing significant morbidity and mortality. Unraveling the molecular mechanisms involved in cardiac hypertrophy has thus become a major focus of research towards developing rational pharmacological approaches for preventing pathological changes associated with this disease.

Among various transducers of intracellular signaling pathways implicated in hypertrophic response, the role of calcineurin (Ca²⁺ - calmodulin dependent protein phosphatase) in cardiac hypertrophy is rapidly gaining importance in view of the well-recognized significance of reversible protein phosphorylation in hypertrophy (17, 26) and the specific ability of calcineurin

inhibitors such as cyclosporine A (CsA) and tacrolimus (FK 506) to reverse cardiac hypertrophy (9). However, the known immunosuppressive and nephrotoxic effects of such inhibitors limit their use as a prophylactic measure for cardiac hypertrophy. It has also been shown that some patients undergoing treatment with CsA may develop hypertension and hypertrophy (11) and that CsA prevents cardiac hypertrophy caused only by certain stimuli (14). The vulnerability of Fe³⁺ in the bimetallic active centre of calcineurin to reactive oxygen species (28) offers an opportunity to evolve alternate strategies for controlling cardiac hypertrophy by using antioxidants. We thus examined the effect of various dietary antioxidants on calcineurin activity and report here the potential benefits of eugenol to alleviate isoproterenol -induced cardiac hypertrophy.

MATERIALS AND METHODS

Animal studies

The studies were conducted with approval of the Ethics Committee of the BARC, Mumbai, India. Male Wistar rats (10 weeks old, weighing 350-400g, n=8 for each group) were categorized into various groups and intraperitoneally injected with either eugenol or isoproterenol for a period of ten days. Different animal groups included Group I-Controls: injected

Corresponding Author:

Prof. C. Subramanyam,
Department of Biochemistry, Osmania University,
Hyderabad 500 007, INDIA,
E-mail : csubramanyam@hotmail.com

daily with dimethyl sulfoxide (DMSO, 0.003% in phosphate buffered saline, PBS); Group II- Isoproterenol treated: injected with ($\pm$) isoproterenol (IP) hemisulphate (Sigma; 1mg/kg body weight/day) dissolved in PBS; Group III- Eugenol treated: injected with eugenol (Sigma; 1 and 10 mg/kg body weight/ twice a day, every 12 hrs) dissolved in 0.003% DMSO; Group IV- Eugenol + IP treated: injected with isoproterenol at 1mg/ kg body weight/day in combination with eugenol at different concentrations of 1mg/kg body weight/ twice a day for a period of ten days. Eugenol was administered to the last group of animals three days prior to injecting isoproterenol. The animals were maintained on standard pelleted diet and water *ad libitum*. After the experimental period, the animals were sacrificed by cervical dislocation. The hearts were dissected, rinsed with PBS, frozen in liquid nitrogen and stored at -20°C till further experimentation. Single cell suspensions of ventricular tissue were obtained essentially as per the method of Miyashita *et al* (15), involving digestion of the minced ventricular tissue in PBS containing collagenase (0.5 mg/ml) for 15 minutes followed by centrifugation ($550 \times g$). To measure TUNEL positive cells and intracellular reactive oxygen species, cells were suspended in PBS to a final density of 0.4×10^6 cells/ ml. Ventricular homogenates were also prepared in liquid nitrogen employing a Tris buffer (5 mM, pH 7.5) containing 0.1 mM NaCl, 5mM dithiothreitol, 1mM EDTA, 1mM phenyl methyl sulfonyl fluoride, 5 $\mu\text{g}/\text{mL}$ pepstatin, 5 $\mu\text{g}/\text{mL}$ leupeptin and 5 $\mu\text{g}/\text{mL}$ aprotinin. The homogenate was clarified by centrifugation ($15,000 \times g$ for 30 minutes at 4°C) and the supernatant was used for further analysis.

Measurement of heart rate

Heart rate of the experimental animals was measured by electrocardiography (BPL India, Cardiart 108T/MK-VI). The legs of restrained rats were immersed in separate Petri dishes and recordings were obtained from electrodes placed near their legs. Measurements were recorded at 25mm per second at a sensitivity at $1\text{mV}=10\text{mm}$. The numbers of QRS complexes that appear over a 60 second period were considered to calculate heart rate.

Determination of reactive oxygen species contents

Oxidation of a fluorogenic probe (2', 7' dichlorodihydrofluorescein diacetate) by reactive oxygen species was monitored essentially as described by Cao *et al.*, (2). Ventricular cell suspensions (2×10^4) were incubated with dichlorodihydrofluorescein diacetate ($10\mu\text{M}$) for ten minutes at $28 \pm 1^{\circ}\text{C}$ in the dark. Release of the fluorescent

dichlorofluorescein was monitored by steady state fluorimetry ($\lambda_{\text{ex}} = 490\text{nm}$, $\lambda_{\text{em}} = 520\text{nm}$, LS 50 B fluorimeter, Perkin Elmer, USA).

TUNEL assay

DNA fragmentation in isolated ventricular cells, indicative of apoptotic cell death, was detected *in situ* employing the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay using a commercial kit (Roche Diagnostics, Mannheim, Germany) as described by Kang *et al* (8). Briefly, air-dried cell samples were fixed on glass slides with freshly prepared p - formaldehyde solution (4% in PBS, pH 7.4) for 1 hour at $15 - 25^{\circ}\text{C}$. The slides were then rinsed in PBS and incubated in a permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 minutes on ice. After rinsing the slides with PBS, they were treated with the reaction mixture containing fluorescein-labeled dUTP and terminal deoxyribonucleotidyl transferase as per the manufacturer's instructions and incubated in a humidified chamber for 60 min at 37°C in the dark. Samples were then analyzed under a fluorescence microscope (Optiphot 2, Nikon, Japan) to determine the number of apoptotic cells.

Estimation of glutathione, calmodulin and calcineurin contents

Glutathione concentrations were measured as per Hissin and Hilf (5) involving the reaction of oxidized (GSSG) as well as reduced glutathione (GSH) with o-phthalaldehyde to yield a fluorescent complex that can be measured ($\lambda_{\text{ex}} = 350\text{nm}$, $\lambda_{\text{em}} = 420\text{nm}$). Calmodulin (CaM) or calcineurin (CaN) contents were determined by competitive ELISA methods employing specific monoclonal antibodies as described earlier (19). Suitable calibration curves were obtained by employing bovine brain CaM (1-25 ng) or CaN (1-500ng) as reference standards.

Enzyme assays

Calcineurin activity was assayed either in human sera (obtained with individual consent from healthy volunteers aged 25 ± 2.5 years) or in ventricular homogenates involving the assay of trifluoperazine-sensitive phosphatase activity using p-nitro phenyl phosphate as the substrate (19). Units of calcineurin activity are in terms of μmoles of p-nitro phenol released from the substrate per minute per deciliter (in case of serum samples) or per mg protein (for ventricular homogenates). Protein kinase C activity was assayed by measuring the incorporation of ($\gamma\text{-}^{32}\text{P}$) ATP into H_1 histone in

the presence and absence of phosphatidyl serine (a specific activator of protein kinase C) as per the method described by Terzian and Rubin (27). One unit of the enzyme is defined in terms of pmoles of (γ - 32 P)-ATP incorporated into the substrate per minute per mg protein. Superoxide dismutase activity was assayed by measuring the ability of the enzyme to inhibit superoxide anion - dependent auto-oxidation of pyrogallol as described by Marklund and Marklund (12). One unit of enzyme activity is the amount required to inhibit auto-oxidation of pyrogallol by 50%. Glutathione peroxidase (GPx) was assayed as per the method of Martinez *et al* (13) involving degradation of cumene hydroperoxide by the enzyme accompanied by oxidation of GSH to GSSG. The fluorescence of the complex formed between GSSG and o-phthalaldehyde was measured in a fluorimeter (λ_{ex} = 350 nm, λ_{em} = 420 nm, LS 50 B fluorimeter, Perkin Elmer, USA). One unit of glutathione peroxidase represents μg of oxidized glutathione formed per mg protein.

Statistics

Data points are presented as Mean $\pm$ SD and were analyzed by 1-way ANOVA between the indicated groups using the Bonferroni multiple comparison test when appropriate or by Student's t-test (25), employing SPSS 10.0 software package (SPSS South Asia).

RESULTS

Isoproterenol activates and eugenol inhibits serum calcineurin activity *in vitro*

Results depicted in Table 1 indicate that trifluoperazine-sensitive calcineurin activity, but not the trifluoperazine-insensitive phosphatase activity of serum, is much more sensitive to isoproterenol. *In vitro* presence of isoproterenol (0.1 mM) in serum samples caused a 2.4 fold increase in calcineurin activity. Even though it is not clear as to why isoproterenol increases calcineurin activity, its ability to do so under *in vitro* conditions is reminiscent of similar observations made *in vivo* earlier with cultured cardiomyocytes (29). Individual presence of eugenol or curcumin or thymol (0.1 mM) in serum samples decreased calcineurin activity to variable extents of 71%, 68% and 48% respectively, indicating eugenol to be the most effective anti-calcineurin agent. When included in the reaction mix along with isoproterenol (0.1 mM each), eugenol resulted in 85.6%, inhibition of serum calcineurin activity as compared to 74.1 and 72.2% inhibition caused by thymol and curcumin respectively.

Eugenol decreases isoproterenol - induced oxidative

Table 1.
Influence of isoproterenol and antioxidants on trifluoperazine-sensitive calcineurin activity and trifluoperazine insensitive phosphatase activity.

Assay conducted in presence of	TFP sensitive calcineurin activity	TFP Insensitive phosphatase activity
Control (No additions)	13.03 $\pm$ 1.3	55.34 $\pm$ 1.9
Isoproterenol (IP)	31.36 $\pm$ 0.7**	16.47 $\pm$ 0.78*
Eugenol	3.71 $\pm$ 0.58**	27.51 $\pm$ 3.5*
Thymol	4.2 $\pm$ 0.2**	16.02 $\pm$ 1.2**
Curcumin	6.79 $\pm$ 1.04*	21.63 $\pm$ 2.0*
IP + Eugenol	4.5 $\pm$ 0.19**	21 $\pm$ 2.9*
IP + Thymol	8.7 $\pm$ 1.4*	8.88 $\pm$ 2.0*
IP + Curcumin	8.1 $\pm$ 1.5*	20.4 $\pm$ 0.4*

Calcineurin activity was assayed in serum samples of healthy volunteers (~ 500 μg protein) in presence of various compounds (0.1 mM). Units of activity are in terms of μmoles of p-nitrophenol released/minute/dL. Activities insensitive to the presence of 150 μM trifluoperazine (TFP) in the reaction mixture were obtained by deducting the TFP sensitive activity of calcineurin from total phosphatase activity assayed. Mean $\pm$ SD values of at least three separate assays using Student's t test are represented. p ** < 0.001 and * <0.05 are given in comparison to controls. p ** < 0.001 and * <0.05 of the last three groups are given in comparison to Isoproterenol treated group (IP treated).

stress and apoptosis in rats

We next examined the influence of eugenol on isoproterenol - induced oxidative stress and apoptosis in rats. Intraperitoneal administration of isoproterenol (1 mg/kg body weight/day) over a period of 10 days caused marked cardiac hypertrophy in rats (Fig. 1). The increased heart size caused by isoproterenol was accompanied with a 64% increase in the ratio of heart weight/total body wt and 20% increase in heart rate (Data not shown). Isoproterenol administration also induced significant (> 10 fold) increase in the number of TUNEL positive cells, indicative of apoptosis. It also caused a 2.6 - fold increase in reactive oxygen species content in ventricular cells and a 50% decrease in total glutathione content without altering the ratio between oxidized and reduced glutathione contents. Such changes were accompanied with a 2-fold increase in activity of superoxide dismutase and a moderate (36%) increase in glutathione peroxidase activity (Table 2).

The ability of eugenol, examined over a concentration range of 1-100 mg/kg body wt, to ameliorate isoproterenol-induced changes was subsequently evaluated. Administering eugenol alone did not cause any change in the observed parameters mentioned above. Since eugenol was effective in this regard even at a low concentration of 1 mg/kg body wt, all further

Fig 1. Influence of isoproterenol and eugenol on heart size. Wistar rats were subjected to different treatments for a period of 10 days as described in Materials and Methods. The photograph depicts representative heart size of various groups of animals viz., 1. Controls 2. Treated with isoproterenol (1 mg/kg body wt/day) 3: Treated with eugenol alone (1 mg/kg body wt, twice a day) 4. Treated with eugenol (1 mg/kg body wt, twice a day) 3 days prior to simultaneous treatment with isoproterenol (1 mg/kg body wt/day) and eugenol (1 mg/kg body wt, twice a day) 5. Treated with eugenol (100 mg/kg body wt) 3 days prior to simultaneous treatment with isoproterenol (1 mg/kg body wt/day) and eugenol (100 mg/kg body wt, twice a day)

experiments were conducted with eugenol at this concentration. Simultaneous administration of eugenol at this concentration along with isoproterenol not only reversed cardiac hypertrophy, but also completely abolished isoproterenol - induced oxidative stress and decreased TUNEL positive cells by about 50%. When eugenol was administered twice a day (1 and 10 mg/kg body weight) it did not affect the gross anatomy of the heart or parameters indicative of oxidative stress (Fig 1 and Table 2). On the contrary, administering eugenol twice a day along with isoproterenol

restored isoproterenol-induced changes even at the lowest concentration examined (1 mg/kg body wt). From the data presented in Table 2, it may be noted that indices such as the heart wt/ body wt, cellular ROS and total glutathione contents were restored to control values when eugenol was administered to rats in combination with isoproterenol. In this regard it is significant that isoproterenol induces inflammation at a very early stage after administration. Since eugenol has been reported to have anti-inflammatory effects (23), the ability of eugenol to inhibit fibrosis is also to be considered while observing its effect on decreasing the heart wt/body wt.

Eugenol restores enhanced cardiac calcineurin and protein kinase C activity caused by isoproterenol

Administration of isoproterenol caused 221% increase in calcineurin activity and 187% increase in protein kinase C activity in ventricular tissue. These activities were not altered upon administration of eugenol alone. On the contrary, combined administration of isoproterenol along with eugenol normalized cardiac activities of calcineurin and protein kinase C. In order to ascertain whether the observed increase in calcineurin activity was also associated with concomitant increase in its content, its cardiac contents were quantitated. While isoproterenol caused a 44% increase in calcineurin content, it was relatively unaffected by eugenol alone. On the contrary, the combined administration of eugenol and isoproterenol (at dosages mentioned above) restored calcineurin content of ventricular tissues to control values. However, there was no concomitant increase in the content of calmodulin, an activator of calcineurin, under such conditions

Table 2
Reversal of isoproterenol-induced changes in rat hearts by eugenol.

Administration with Cardiac Parameter Studied	Control	IP	Eugenol	IP + Eugenol (No treatment)
Heart wt/Body wt (Ratio)	3.37 ± 0.25	5.54 ± 0.39**	3.45 ± 0.26	3.9 ± 0.10*
ROS content (RFI)	59.37 ± 0.29	155.5 ± 3.11**	36 ± 2.0**	60.65 ± 0.18
TUNEL positive cells (%)	2.86 ± 0.22	30.53 ± 1.13**	3.20 ± 1.2	16.23 ± 0.90**
Total Glutathione (nmol/mg protein)	15.56 ± 2.6	7.23 ± 2.29*	15.66 ± 0.21	14.83 ± 2.05
GSH/GSSG (Ratio)	0.9 ± 0.02	1.19 ± 0.29	0.92 ± 0.04	0.97 ± 0.34
SOD activity (Units)	2.73 ± 0.57	5.63 ± 1.13**	2.26 ± 0.03	2.3 ± 0.18
GPx activity (Units)	247.1 ± 7.56	350 ± 9.19**	247 ± 7.21	239.1 ± 9.97

Ventricular tissue obtained from Wistar rats (n= 8) were processed and analyzed for various parameters as given in Materials and Methods. Indicated administrations are IP= Isoproterenol treatment for 10 days (1 mg/kg body wt/day); Eugenol = (1 mg/kg body wt/ 2 x day); IP + Eugenol = combined administration with isoproterenol (1 mg/kg body wt/day) and eugenol (1 mg/kg body wt/ 2 x day). Mean± SD values of at least three separate assays using 1-way ANOVA are represented. RFI= Relative Fluorescence intensity. p** < 0.001 and * < 0.05 are given in comparison to control values.

TABLE 3
INFLUENCE OF ISOPROTERENOL AND EUGENOL ON CARDIAC CALCINEURIN AND PROTEIN KINASE C ACTIVITIES.

Administration with Cardiac Parameter Studied	Control	IP	Eugenol	IP + Eugenol (No treatment)
Calmodulin content (ng/mg protein)	72.65 ± 6.15	74.03 ± 3.97	75.5 ± 4.95	78.0 ± 2.82
Calcineurin content (ng/mg protein)	278.4 ± 17.82	402.6 ± 11.31**	248.3 ± 38.80	304.7 ± 8.34
Calcineurin activity (Units)	26.8 ± 4.24	59.3 ± 1.06**	24 ± 2.0	30.1 ± 3.51
Protein kinase C activity (Units)	4.7 ± 0.20	8.8 ± 0.81**	4.2 ± 0.17	4.2 ± 0.31

Calcineurin and Protein kinase C activities in rat ventricular extracts were assayed as described in Materials and Methods. Indicated administrations are IP = Isoproterenol treatment for 10 days (1 mg/kg body wt/day); Eugenol = (1 mg/kg body wt/ 2 x day); IP + Eugenol = combined administration with isoproterenol (1 mg/kg body wt/day) and eugenol (1 mg/kg body wt/ 2 x day). Mean ± SD values of at least three separate assays using 1-way ANOVA are represented. $p^{**} < 0.001$ is given in comparison to control values.

(Table 3).

DISCUSSION

Recent years have witnessed accumulating evidence for the involvement of oxidative stress in cardiac diseases including cardiac hypertrophy (22). Isoproterenol leads to oxidative stress which contributes significantly to the pathophysiology of the disease (3). Of the various events involved in signal transduction, much importance is now being ascribed to calcineurin activity in view of the demonstrated ability of calcineurin inhibitors to ameliorate cardiac hypertrophy (9, 16). However, the immunosuppressive effects of these agents limit their use as a prophylactic measure for cardiac hypertrophy. Thus, we undertook the present investigation to identify natural antioxidants devoid of immunosuppressive functions, but capable of inhibiting calcineurin activity. Initial experiments conducted *in vitro* with serum calcineurin revealed that antioxidants can reverse enhanced enzyme activity brought about by isoproterenol. Such an observation is in accord with earlier reports, which ascribed the ability of isoproterenol to induce cardiac hypertrophy by promoting calcineurin activity under *in vivo* conditions (29). Importantly, among the various antioxidants examined, we could identify eugenol as the more potent natural antioxidant capable of countering the effect of isoproterenol (Table 1).

In continuation of these observations made with serum samples, the *in vivo* effects of eugenol in preventing isoproterenol induced cardiac hypertrophy were examined. Results presented in Table 2 denote that isoproterenol administration resulted not only in increased heart size, heart

rate and TUNEL positive cells indicative of apoptosis, but also increased oxidative stress in ventricular tissue as evidenced by (i) increased ROS contents (ii) increased activities of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase and (iii) decreased total glutathione content in ventricular tissue. The observed increase in free radical scavenging enzymes (superoxide dismutase and glutathione peroxidase) indicate that oxidative stress caused by isoproterenol may up-regulate the activity of antioxidant enzymes to facilitate rapid removal of the accumulated reactive oxygen species. In addition, the ability of isoproterenol to enhance the number of apoptotic cells in ventricular tissue is in agreement with similar results obtained upon β -adrenergic stimulation (24). The observed increase in cardiac protein kinase C activity under isoproterenol-induced cardiac hypertrophy is in agreement with earlier studies indicating that all hypertrophic stimuli activate one or more phospholipases with liberation of diacylglycerol and subsequent activation of protein kinase C (1). The observed increase of cardiac calcineurin activity is also in conformity with earlier reports indicating that isoproterenol activates extra cellular signal regulated protein kinases through calcineurin (29).

Different possibilities are to be considered in understanding the cardio-protective effects of eugenol (4-allyl-2-methoxy phenol), a naturally occurring antioxidant present as the active principle of cloves (*Syzygium aromaticum*) and other medicinal and aromatic plants such as *Ocimum sanctum* and *Pimenta racemosa*. In view of its nonmutagenic and noncarcinogenic properties, eugenol is generally regarded as safe by the FAO with an acceptable daily intake up to 2.5 mg/kg body wt in humans (4). Pharmacokinetic and metabolic studies also

reveal that eugenol exerts its biological effects through its antioxidant properties including the inhibition of lipid peroxidation (7, 20). At a physiological level, eugenol might function as an antagonist of β -adrenergic stimulation caused by isoproterenol. Although derivatives of eugenol, such as isoeugenolol and eugenodilol, have been reported to function as antagonists/blockers of β_1 adrenergic receptors (6, 10), no such effects have been ascribed to eugenol. The demonstrated ability of calcineurin inhibitors to decrease calcineurin gene expression (18) and the reported effect of antioxidants on calcineurin-mediated activation of transcriptional factors (21) point to other possible modes through which antioxidants may prevent cardiac hypertrophy. While the present study strongly favors the use of antioxidants such as eugenol in preventing and treating cardiac hypertrophy, further investigations are required to identify more potent natural inhibitors of calcineurin for their possible use towards preventing cardiac hypertrophy.

ACKNOWLEDGEMENTS

This work was supported through a research grant awarded to CS by the Department of Science and Technology (SP/SO/A69/97).

REFERENCES

- Braun, M., Simonis, G., Birkner, K., Pauke, B. and Strasser, R.H (2003) Regulation of protein kinase C isozyme and calcineurin expression in isoproterenol induced cardiac hypertrophy. *J. Cardiovasc. Pharmacol.* 41, 946-954.
- Cao, Z., Tsang, M., Zhao, H. and Li, Y (2003) Induction of endogenous antioxidants and phase 2 enzymes by α -lipoic acid in rat cardiac H9C2 cells: protection against oxidative injury. *Biochem. Biophys. Res. Commun.* 310, 979-985.
- Chattopadhyay, A., Biswas, S., Bandyopadhyay, D., Sarkar, C. and Datta, A.G (2003) Effect of isoproterenol on lipid peroxidation and antioxidant enzymes of myocardial tissue of mice and protection by quinidine. *Mol. Cell. Biochem.* 245, 43-49.
- FAO (1982) Evaluation of certain food additives and contaminants. Technical Report Series No. 20. FAO/WHO Expert Committee on food additives.
- Hissin, P.J. and Hilf, R (1976) A fluorimetric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* 74, 214-226.
- Huang, Y.C., Wu, B.N., Lin, Y.T., Chen, S.J., Chiu, C.C., Cheng, C.J. and Chen, I.J (1999) Eugenodilol: a third-generation beta-adrenoceptor blocker, derived from eugenol, with alpha-adrenoceptor blocking and beta2-adrenoceptor agonist-associated vasorelaxant activities. *J. Cardiovasc. Pharmacol.* 34, 10-20.
- Jayashree, T. and Subramanyam, C (1999) Antiaflatoxicogenic activity of eugenol is due to inhibition of lipid peroxidation. *Lett. Appl. Microbiol.* 28, 179-183.
- Kang, Y.J., Zhou, Z.X., Wu, H., Wang, G.W., Saari, J.T. and Klein, J.B (2000) Metallothionein inhibits myocardial apoptosis in copper-deficient mice: role of atrial natriuretic peptide. *Lab. Invest.* 80, 745-757.
- Leinwand, L.A (2001) Calcineurin inhibition and cardiac hypertrophy: A matter of balance. *Proc. Natl. Acad. Sci. USA.* 98, 2947-2949.
- Lin, Y.T., Wu, B.N., Horng, C.F., Huang, Y.C., Hong, S.J., Lo, Y.C., Cheng, C.J. and Chen I.J (1999) Isoeugenolol: A selective beta 1-drenergic antagonist with tracheal and vascular smooth muscle relaxant properties. *Jpn. J. Pharmacol.* 80, 127-136.
- Lipkin, G.W., Tucker, B., Giles, M. and Raine, A.E (1993) Ambulatory blood pressure and left ventricular mass in cyclosporin and not cyclosporin treated renal transplant recipients. *J. Hypertens.* 11, 439-442.
- Marklund, S. and Marklund, G (1974) Involvement of the superoxide anion radical in the auto oxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* 47, 469-474.
- Martinez, J.I.R., Launay, J.M. and Dreux, C (1979) A sensitive fluorimetric microassay for glutathione peroxidase activity. Application to human blood platelets. *Anal. Biochem.* 98, 154-159.
- Meguro, T., Hong, C., Asai, K., Takagi, G., McKinsey, T.A., Olson, E.N. and Vatner, S.F (1999) Cyclosporine attenuates pressure overload hypertrophy in mice while enhancing susceptibility to decompensation and heart failure. *Circ. Res.* 84, 735-740.
- Miyashita, T., Takeishi, Y., Takahashi, H., Kato, S., Kubota, I. and Tomoike, H (2001) Role of calcineurin in insulin-like growth factor-1-induced hypertrophy of cultured adult rat ventricular myocytes. *Jpn. Circ. J.* 65, 815-819.
- Molkentin, J., Lu, J., Antos, C., Markham, B., Richardson, J., Robbins, J., Grant, S. and Olson, E.N (1998) A calcineurin dependent transcriptional pathway for cardiac hypertrophy. *Cell.* 93, 215-228.

17. Molkenstin, J.D (2000) Calcineurin and beyond: Cardiac hypertrophy signaling. *Circ. Res.* 87, 731-738.
18. Nagata, K., Somura, F., Obata, K., Odashima, M., Izawa, H., Ichihara, S., Nagasaka, T., Iwase, M., Yamada, Y., Nakashima, N. and Yokota, M (2002) AT1 receptor blockade reduces cardiac calcineurin activity in hypertensive rats. *Hypertension.* 40, 168-74.
19. Padma, S. and Subramanyam, C (1999) Extracellular calcineurin: Identification and quantitation in serum and amniotic fluid. *Clin. Biochem.* 32, 491-494.
20. Pandey, B.N. and Mishra, K.P (2003) Modification of thymocytes membrane radio-oxidative damage and apoptosis by eugenol. *J. Environ. Pathol. Toxicol. Oncol.* in Press;
21. Sacconi, S., Sacconi, A., Varesio, L., Ghosh, P., Young, H.A. and Sica, A (1999) Divergent effects of dithiocarbamates on AP-1-containing and AP-1-less NFAT sites. *Eur. J. Immunol.* 29, 1194-1201.
22. Sawyer, D.B., Siwik, D.A., Xiao, L., Pimentel, D.R., Singh, K. and Colucci, W.S (2002) Role of oxidative stress in myocardial hypertrophy and failure. *J. Mol. Cell. Cardiol.* 34, 379 {INCLUDEPICTURE "http://www.pubmedcentral.nih.gov/corehtml/pmc/pmcents/ndash.gif" /* MERGEFORMATINET} 388.
23. Sharma, J.N., Srivastava, K.C. and Gan, E.K (1994) Suppressive effects of eugenol and ginger oil on arthritic rats. *Pharmacology.* 49, 314-318.
24. Shizukuda, Y., Buttrick, P.M., Geenen, D.L., Borczuk, A.C., Kitsis, R.N. and Sonnenblick, E.H (1998) beta-adrenergic stimulation causes cardiocyte apoptosis: influence of tachycardia and hypertrophy. *Am. J. Physiol.* 275, H961-H968.
25. Snedecor, G.W. and Cochran, W.G. (1986) Shortcut and non-parametric methods. In: *Statistical methods*, Eds. Snedecor, G.W. and Cochran, W.G, Oxford and IBH, New Delhi, 120-132.
26. Sugden, P.H (2001) Signalling pathways in cardiac myocyte hypertrophy. *Ann. Med.* 33, 611-622.
27. Terzian, A.R. and Rubin, R.P (1993) Translocation of the α -isozyme of protein kinase C during stimulation of rat parotid acinar cells by phorbol ester and carbachol. *Archives. Oral. Biol.* 38, 1051-1056.
28. Wang, X., Culotta, V.C. and Klee, C.B (1996) Superoxide dismutase protects calcineurin from inactivation. *Nature.* 383, 434-437.
29. Zou, Y., Yao, A., Zhu, W., Kudoh, S., Hiroi, Y., Shimoyama, M., Uozumi, H., Kohmoto, O., Takahashi, T., Shibasaki, F., Nagai, R., Yazaki, Y. and Komuro, I (2001) Isoproterenol activates extracellular signal-regulated protein kinases in cardiomyocytes through calcineurin. *Circulation.* 104, 102-108.