

# Cotinine Overcome the Decrease of Prostacyclin Formation Induced by Nicotine after Sympathetic Stimulation in Microsomes from Isolated Rabbit Heart

G. Ammar<sup>1</sup>, R. Nembr<sup>2</sup>, Z. Dagher<sup>1</sup>, S. Khairallah<sup>1</sup>, R. Chahine<sup>1</sup>

<sup>1</sup>Laboratory of physiology, Faculty of medical sciences, Lebanese University, Beirut (Lebanon).

<sup>2</sup>Department of Surgery, Maimonides Medical Center, Brooklyn NY, USA .

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## ARTICLE INFO

### Article history:

Received on: 08/03/2013

Revised on: 04/04/2013

Accepted on: 14/06/2013

Available online: 30/06/2013

### Key words:

Isolated heart, Sympathetic stimulation, Nicotine, Cotinine, Prostacyclin.

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## ABSTRACT

We studied the effects of cotinine, the major metabolite of nicotine, on nicotine-induced decrease in prostacyclin (PGI<sub>2</sub>) synthase activity of microsomes from isolated rabbit heart after sympathetic stimulation. Rabbit hearts were isolated along with their sympathetic nerves and perfused in accordance to the Langendorff method. Hearts were randomly divided into 10 groups treated with cotinine, either during sympathetic stimulation or prior to nicotine administration. PGI<sub>2</sub> formation in cardiac microsomes was assessed by radioimmunoassay. The results showed that sympathetic stimulation increased PGI<sub>2</sub> synthase activity of heart microsomes by 32 %. Nicotine dose dependently decreased PGI<sub>2</sub> while cotinine increased it. In addition, perfused before nicotine, cotinine prevented the PGI<sub>2</sub> decreasing effect of nicotine. The results suggest that pre-treatment with cotinine can be involved in the modulation of nicotine effects on PGI<sub>2</sub> in hearts subjected to sympathetic stimulation.

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## INTRODUCTION

Smoking and tobacco use are significant risk factors for cardiovascular diseases that are considered the major causes of morbidity and mortality in the world (Sorlie *et al.* 2012). The link between smoking and coronary artery diseases is well established however the mechanism of action has not been fully elucidated. Accumulating evidence attributes the triggering and the persistence of smoking dependence to a cascade of events initiated by nicotine (Machii *et al.* 2012). Interestingly, nicotine is metabolized mainly into cotinine, by hepatic cytochromes P-450 oxidation, and possesses a longer half-life than its precursor (Benowitz *et al.* 2009).

It has been established that Thromboxane (TXA<sub>2</sub>) and Prostacyclin (PGI<sub>2</sub>) are implicated in the pathophysiology of cardiovascular diseases as they play an important role in homeostasis characterized by the local interaction with platelets and the vascular endothelial layer.

Hence, any disruption of the equilibrium affecting the biosynthesis or the release of eicosanoids could mechanistically underlie to several disorders, such as myocardial ischemia (Iñiguez *et al.* 2008). These include (1) functional transformation of arachidonic acid (AA) into PGH<sub>2</sub> then to TXA<sub>2</sub> secondary to the action of cyclooxygenase and thromboxane synthase in platelets; and (2) production of PGI<sub>2</sub> by prostacyclin synthase. TXA<sub>2</sub> is a vasoconstrictor and stimulates aggregation while PGI<sub>2</sub> is a vasodilator with anti-aggregation effects (Lüscher *et al.* 1993).

Previous work from our laboratory (Chahine *et al.* 1991, Nembr *et al.* 2003) has reported an inhibitory effect of nicotine on the PGI<sub>2</sub> synthase activity in aortic microsomes. Furthermore, it has also been suggested that the myocardium possesses a TXA<sub>2</sub> synthase activity that can be modulate by ischemia. In the present study, we investigated the effects of nicotine and cotinine separately on the production of PGI<sub>2</sub> after sympathetic stimulation, and the effects of cotinine followed by nicotine administration on the production of PGI<sub>2</sub> after sympathetic stimulation. We hypothesized that the metabolism of nicotine to cotinine may reverse the adverse effects of its precursor on the production of PGI<sub>2</sub> by cardiac microsomes following sympathetic stimulation.

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\* Corresponding Author

Prof. R. Chahine, Laboratory of physiology,

Faculty of medical sciences, Lebanese University BP 3 Hadath campus, Beirut Lebanon. Phone 961 3 534165

## MATERIALS AND METHODS

### Isolated nerve–heart preparation

Male rabbits (*Oryctolagus cuniculus*) weighing  $1.5 \pm 0.2$  kg were used for this study. After anesthesia with pentobarbital (60mg /kg), hearts were rapidly isolated but maintained in the thorax and retrogradely perfused according to Langendorff mode via the aorta at constant pressure (60 cm H<sub>2</sub>O) with a tyrode solution, in mM (NaCl: 136.9, KCl: 2.7, CaCl<sub>2</sub>: 1.8, MgCl<sub>2</sub>: 1.0, NaHCO<sub>3</sub>: 11.9, NaH<sub>2</sub>PO<sub>4</sub>: 0.4, Glucose: 0.6), pH: 7.4, to: 37° C (Chahine *et al.* 1991). In addition, the region extending from the neck to thorax was dissected from surrounding tissues in a manner to isolate the sympathetic nerves. The heart-nerve preparation was then transferred to the perfusion apparatus; the nerves were introduced in glass tubes equipped with 2 electrodes connected to an electric stimulator (Harvard Apparatus) and superfused with the tyrode solution. All procedures were undertaken in accordance with the current Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health

### Experimental protocol

The experiments started approximately 20 min after setting up the perfusion when the physiological parameters were stabilized. Hearts with irregularities were discarded. Therefore, hearts that have responded to the selection requirements were randomly separated into 10 groups, n = 6 – 8 each:

- Group 1: Control hearts perfused with a standard tyrode solution
- Group 2 and 3 : Hearts submitted to 5 or 10 min sympathetic stimulation (2 Hz)
- Group 4 and 5: Hearts treated with nicotine (0.1 or 10 μM)
- Group 6 and 7: Hearts treated with cotinine (1 or 100 μM)
- Group 8: Hearts treated with nicotine (10 μM) during 10 min sympathetic stimulation
- Group 9: Hearts treated with cotinine (100 μM) during 10 min sympathetic stimulation
- Group 10: Hearts treated with cotinine (100 μM), 10 min before and during 10 min nicotine perfusion (10 μM) and sympathetic stimulation.

Nicotine and cotinine perfusion was done using a pump with a constant rate of 1 ml/min in order to prevent any sensible perturbation of the perfusion pressure. At the end of the experiments, the hearts were rapidly immersed in nitrogen liquid and stored at -80 °C until assay. These hearts will serve for the preparation of the microsomes.

### Microsomes preparation

In order to prepare the microsomes, the frozen hearts were thawed then dissected to remove adherences to the tissues. They were ground and homogenized in cold phosphate buffer 0.1 mmol, pH 8.0. The homogenate was centrifuged at 12000 g for 10 min. The supernatant was filtered through gauzes then centrifuged at 105000 g for 60 min at 0 °C and the resulting pellet was homogenized in phosphate buffer and lyophilized.

### Determination of PGI<sub>2</sub> synthase activity of the myocardium

Myocardial microsomes were used as a source of PGI<sub>2</sub> synthase. They were incubated in vitro with the AA. These microsomes were preincubated during 10 min at 37 °C then AA. ( $3 \cdot 10^{-5}$ ) was added and the reaction was arrested by citric acid after 2 min of incubation. 6 keto PGF<sub>1α</sub> formed was extracted 2 times with the diethyl ether, then centrifuged T 600 g during 10 min at +4 °C. The supernatant was then extracted and evaporated till dried under nitrogen current. At the end a phosphate buffer 0.1 M pH 7.4 was added to the extracted product and dosed by (RIA) for 6 keto PGF<sub>1α</sub>. PGI<sub>2</sub> is rapidly metabolized into 6 keto PGF<sub>1α</sub> because of its brief half-life. The concentrations of the substrate and the enzymes are considered to be the optimal concentration chosen giving reproducible results; these concentrations have been chosen indeed after several preliminary experimentations.

### Proteins dosage

Protein of microsomes was determined after Lowry's method modified by Hartree *et al.* (1972) using bovine serum albumin (Merck) as a standard. Then the results were drawn on a graphic depending on the protein concentration (BSA). The presence of the microsomal fraction and the absence of platelets were verified by appropriate microscopy methods.

### Radioimmunoassay technique for 6 keto PGF<sub>1α</sub>.

6-keto PG F<sub>1α</sub> was analyzed by radioimmunoassay techniques developed on the basis of the method of Dray *et al.* (1975). Sample extracts or a standard solution containing 6-keto PG F<sub>1α</sub> (Cayman Chemical) ranging from 10 to 500 pg were dissolved in phosphate buffer. 5,8,9,11, 12,14,15 -[3H] (N) 6-keto PG F<sub>1α</sub>, 120 Ci/mmol from PerkinElmer NEN Radiochemicals were added, followed by the appropriate specific antibody from Institute Pasteur (Paris). The cross reactivity of 6-keto PG F<sub>1α</sub> antibody was 100 % for 6-keto PG F<sub>1α</sub>, 2.8 % for PG F<sub>2α</sub>, 4 % for PG E<sub>2</sub> and less than 0.1 % for PG A<sub>2</sub> and TX B<sub>2</sub>. The final assay volume was 250 μl. Tubes were vortexed, incubated at 22 °C for 1 h, then at 4 °C overnight. The antibody-bound and antibody-free icosanoids were separated using dextran-coated charcoal suspension (5.0 mg Norit Prolabo + 0.5 mg dextran T 70 Pharmacia / 1 ml phosphate buffer at 0°C for 13 min). After centrifugation at 2000 g for 10 min (J 21 B Beckman refrigerated centrifuge), an aliquot of the supernatant was placed in Instagel scintillating fluid (Packard) and counted in a Packard Tricab 2450 liquid scintillation spectrometer. B/Bo was calculated for each sample the concentration of which was determined from a standard curve after logit transformation (Bo: maximal binding in dpm, B: dpm of the various samples). The dilutions used were sufficient to produce a 6-keto PG F<sub>1α</sub> content decreasing within the linear portion of the standard curve (B/Bo = 20 - 80 %). The detection limits of the RIA procedure was 25 pg.

### Statistics

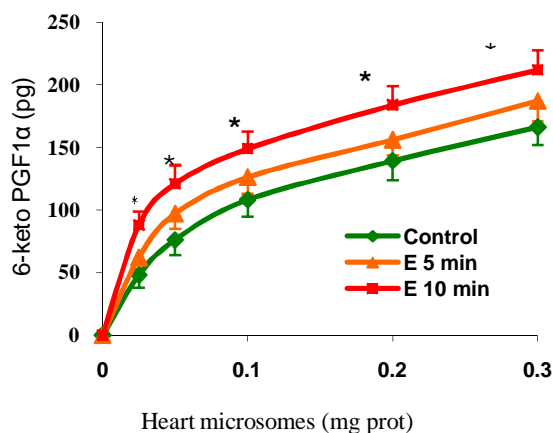
All values were expressed as means ± SEM. Variance analysis designed for repeated measures was carried out under

various experimental conditions between groups.  $p$  values  $< 0.05$  was considered significant. Significance of the results was assessed using Student's  $t$  test for unpaired data using the Bonferroni correction.

## RESULTS

### Effect of sympathetic stimulation on the biosynthesis of PGI<sub>2</sub>

Sympathetic stimulation for 5 min, slightly, but not significantly increased myocardial PGI<sub>2</sub> synthase activity. However, 10 min of sympathetic stimulation significantly ( $p < 0.05$ ) increased this activity (Figure 1). Indeed, heart microsomes from hearts submitted to 10 min sympathetic stimulation produced more 6-keto-PGF<sub>1 $\alpha$</sub> , stable metabolite of the PGI<sub>2</sub>, than those perfused solely by standard tyrode solution.



**Fig. 1.** PGI<sub>2</sub> biosynthesis in vitro of microsomes from isolated rabbit heart submitted to sympathetic stimulation during 5 or 10 min, on.  $n = 6-8$ ,  $*p < 0.05$  (E: electrical stimulation; 6-keto-PGF<sub>1 $\alpha$</sub> : stable metabolite of PGI<sub>2</sub>; pg: picogram)

### Effect of nicotine on the biosynthesis of PGI<sub>2</sub>

Microsomes from hearts perfused with nicotine for 10 min, at concentrations of 0.1  $\mu$ M and 10  $\mu$ M, produced less 6-keto-PGF<sub>1 $\alpha$</sub>  than those not perfused by nicotine ( $p < 0.05$  for the dose of 10  $\mu$ M). Thus, we found a dose dependent nicotine inhibition of PGI<sub>2</sub> synthase activity (Figure 2a).

### Effect of cotinine on the biosynthesis of PGI<sub>2</sub>

Microsomes from hearts perfused with cotinine for 10 min, at concentrations of 1  $\mu$ M and 100  $\mu$ M, produces more 6-keto-PGF<sub>1 $\alpha$</sub>  than those not perfused by cotinine ( $p < 0.05$  for the dose of 100  $\mu$ M). Thus, we found a dose dependent cotinine stimulation of PGI<sub>2</sub> synthase activity (Figure 2b).

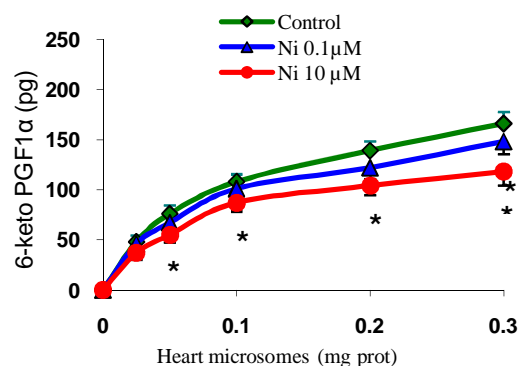
### Effect of nicotine and sympathetic stimulation on the biosynthesis of PGI<sub>2</sub>

Microsomes of hearts perfused by nicotine at a concentration of 10  $\mu$ M during sympathetic stimulation for 10 min, produces less 6-keto-PGF<sub>1 $\alpha$</sub>  than those receiving only sympathetic

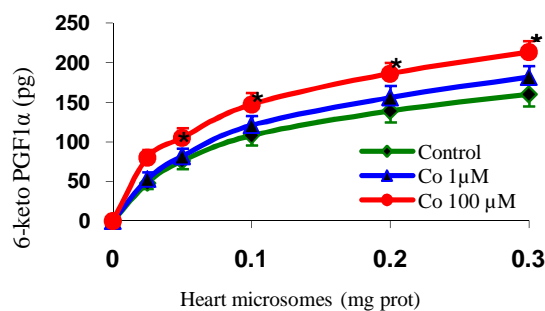
stimulation. Thus, nicotine inhibits the sympathetic effect on PGI<sub>2</sub> synthase activity (Figure 3a).

### Effect of cotinine and sympathetic stimulation on the biosynthesis of PGI<sub>2</sub>

Cotinine perfusion at a concentration of 100  $\mu$ M during 10 min of sympathetic stimulation does not modify the effect of the latter on PGI<sub>2</sub> synthase activity for the production of 6-keto-PGF<sub>1 $\alpha$</sub>  (Figure 3b).

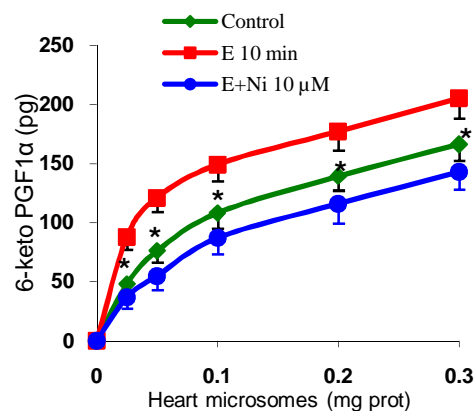


(a)

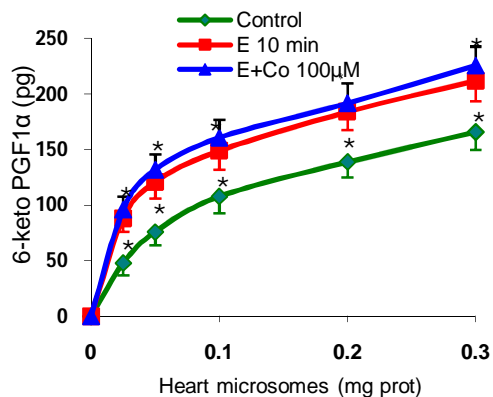


(b)

**Fig. 2:** a) PGI<sub>2</sub> biosynthesis in vitro of microsomes from isolated rabbit heart submitted to nicotine (0.1 and 10  $\mu$ M) perfusion b) PGI<sub>2</sub> biosynthesis in vitro of microsomes from isolated rabbit heart submitted to cotinine (1  $\mu$ M and 100  $\mu$ M) perfusion.  $n = 6-8$ ,  $*p < 0.05$  (Ni: Nicotine; Co: cotinine; 6-keto-PGF<sub>1 $\alpha$</sub> : stable metabolite of PGI<sub>2</sub>; pg: picogram).

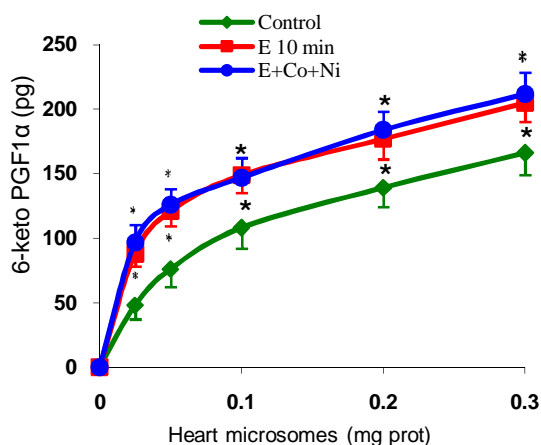


(a)



(b)

**Fig. 3:** a) PGI<sub>2</sub> biosynthesis in vitro of microsomes from isolated rabbit heart submitted to nicotine perfusion (10 μM) during 10 min sympathetic stimulation b) PGI<sub>2</sub> biosynthesis in vitro of microsomes from isolated rabbit heart submitted to cotinine perfusion (100 μM) during 10 min sympathetic stimulation. n = 6-8, \*p < 0.05 (Ni: nicotine; Co: cotinine; 6-keto-PGF<sub>1α</sub>: stable metabolite of PGI<sub>2</sub>; pg: picogram)



**Fig. 4.** PGI<sub>2</sub> biosynthesis in vitro of microsomes from isolated rabbit heart submitted to cotinine perfusion (100 μM) before and during 10 min of nicotine perfusion (10 μM) and sympathetic stimulation. n = 6-8, \*p < 0.05. (E: electrical stimulation; Ni: nicotine; Co: cotinine; 6-keto-PGF<sub>1α</sub>: stable metabolite of PGI<sub>2</sub>; pg: picogram)

#### Effect of cotinine followed by nicotine under sympathetic stimulation on the biosynthesis of PGI<sub>2</sub>

Cotinine perfusion at a concentration of 100 μM for 10 min followed by a perfusion of nicotine at a concentration of 0.1 μM during 10 min of sympathetic stimulation does not modify the PGI<sub>2</sub> synthase activity of heart microsomes for the production of 6-keto-PGF<sub>1α</sub> (Figure 4).

#### DISCUSSION

The main pronounced finding of this study is that pre-existing cotinine could modify responses to acute nicotine effect on prostacyclin formation in a sympathetically stimulated rabbit heart.

Our aim is to study the effects of sympathetic stimulation and drug treatments on PGI<sub>2</sub> formation in the myocardium using heart microsomes as a source of PGI<sub>2</sub> synthase. Since the interventions were done on the isolated heart, the enzyme present in the microsomal fraction should be necessarily modulated, and then we used the heart microsomes as a source of PGI<sub>2</sub> synthase in vitro to assess PGI<sub>2</sub> formation from arachidonic acid. This study is not devoted to know the mechanism by which the products binds to the enzyme but rather to know the final effect on PGI<sub>2</sub> formation. Our approach mimics in vitro the biosynthesis pathway of PGI<sub>2</sub> from arachidonic acid independently from other pathways in the myocardial cell.

Our observation that sympathetic stimulation increases the production of prostacyclin is in agreement with Gonzales *et al.* (1991) and Nebigil *et al.* (1992) who in turn demonstrated an induction of prostacyclin production after noradrenaline binding to the α-adrenergic receptor. However, Ruan *et al.* (1997) found that this effect is mediated by the β-adrenergic receptor stimulation. According to Stewart *et al.* (1984) noradrenaline increases the production of prostacyclin in the rat aorta. Whereas, Neri Serneri *et al.* (1990) found that serum levels of prostacyclin decrease following sympathetic stimulation via repetitive cold stimuli in healthy young men inducing progressive vasoconstriction at a steady maximal norepinephrine concentration. At the cardiovascular regulation level, it will be more favorable to have an increase in the production of prostacyclin (vasodilator and anti-aggregation effect) to limit the vasoconstriction effects of noradrenaline (Gwozdz *et al.* 2007).

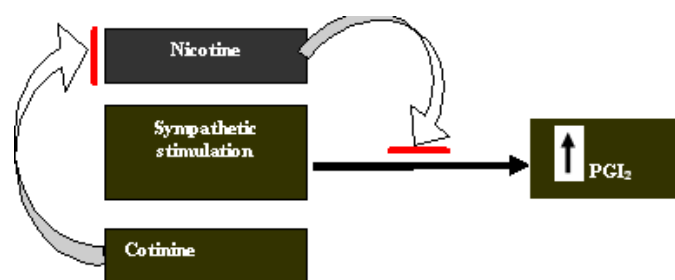
Our results showed also that the perfusion of hearts by nicotine (10 μM) inhibits prostacyclin synthase activity and the stimulatory effect of sympathetic stimulation on the production of prostacyclin by heart microsomes. Inversely, cotinine perfusion at a concentration of 100 μM with or without a subsequent sympathetic stimulation may significantly accelerate this enzyme activity. These findings were similar to the results of prior studies in rabbits (Stoel *et al.* 1982), and consistent with the prior finding that nicotine decreases prostacyclin production at the umbilical cord artery level (Chahine *et al.* 1996). Few studies examined the general effects of cotinine, where it has been shown to alter noradrenalin secretion stimulated by nicotine in the bovine adrenal gland chromafines cells (Vainio *et al.* 1998). Others have demonstrated that cotinine may inhibit endothelium-dependent vascular relaxation of the carotid and coronary arteries (Conklin *et al.* 2001).

The present study added a novel finding to our previous work that cotinine perfusion at a concentration 100 μM followed by nicotine perfusion at a concentration of 10 μM before sympathetic stimulation can overcome the inhibitory effects of nicotine on the prostacyclin synthase production by rabbit heart microsomes. Another important feature of this study is the use of viable sympathetic nerves from the isolated rabbit heart. Such methodology is expected to better simulate the physiological effects of sympathetic stimulation. Moreover, the nicotine and cotinine doses used in the present study exceed those produced by

a smoked cigarette, although, a regular smoker may retain more nicotine and mainly cotinine in blood and other organs. The use of nitrogen liquid and  $-80^{\circ}\text{C}$  storage is also known to conserve tissues and replicate cellular reactions consistently

## CONCLUSION

In conclusion, nicotine inhibits the stimulatory effect of sympathetic stimulation on the prostacyclin production by heart microsomes, while cotinine suppresses such inhibitory effect of nicotine (Figure 5). Thus, cotinine, the main metabolite of nicotine, can be a new marker for testing of therapies aimed at reducing the harmful effects accompanying smoking and tobacco use may be an interesting subject for further studies aiming to reduce the baleful effect of tobacco smoking.



**Fig. 5.** Diagram illustrating prostacyclin production by microsomes from rabbit hearts sympathetically stimulated and perfused by nicotine or cotinine. Thus, cotinine inhibits the inhibition exerted by nicotine on the stimulatory effect of the sympathetic.

## ACKNOWLEDGMENTS

This project was supported by a grant team from the Research Committee, Lebanese University and Lebanese National Council for Scientific Research.

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### How to cite this article:

G. Ammar, R. Nemr, Z. Dagher, S Khairallah, R. Chahine. Cotinine Overcome the Decrease of Prostacyclin Formation Induced by Nicotine after Sympathetic Stimulation in Microsomes from Isolated Rabbit Heart. *J App Pharm Sci.* 2013; 3 (06): 208-212.