Inhibition of PDE4 by low doses of rolipram induces changes in lipid and protein components of mice heart

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Abstract. There is significant increasing interest in phosphodiesterase-4 (PDE4) inhibition in treatment of cardiovascular diseases. Related with this, research has focused on cellular, biochemical, molecular and structural changes in heart tissue induced by PDE4s inhibitors. However, for their clinical applicability additional studies are still needed. Fourier transform infrared spectroscopy offers promising approach to contribute such issue due to its ability in detection the changes in biomolecules. By utilizing this method, we examined the effects of PDE4 inhibition by rolipram at 0.05 mg/kg and 0.1 mg/kg doses on content of lipids and proteins, and fluidity, order and packing of membranes in naive mice heart. In treated groups, there was a significant decrease in unsaturated, saturated lipids, cholesterol esters, fatty acids, phospholipids and triacylglycerols obtained from CH₂, C=O, olefinic=CH, and COO⁻ areas, and CH₂/lipid, C=O/lipid, olefinic=CH/lipid, and COO⁻/lipid ratios. Additionally, olefinic=CH area and olefinic=CH/lipid ratio may suggest decreased lipid peroxidation, confirmed by thiobarbituric acid assay. Also, a higher degree of membrane order, slight increase in membrane fluidity and differences in membrane packing were obtained. Amide I and II areas and RNA/protein ratios showed that variation in protein content is not correlated with applied concentration. Analysis of amide I mode predicted alterations in secondary structures like an increase in random coils and decrease in alpha-helices. Moreover, all groups were successfully discriminated by cluster analysis. The corresponding results may help to understand the potential effects of PDE4 inhibition by rolipram.

Key words: Rolipram — Heart — FTIR — Lipid — Protein — Membrane order/packing/fluidity

Abbreviations: BHT, buthylatedhydroxytoluene; cAMP, cyclic adenosine 3',5'-monophosphate; DMSO, dimethyl sulfoxide; FT-IR, Fourier transform infrared; IR, infrared light; LPEPs, lipid peroxidation end products; MDA, malondialdehyde; PDE4, phosphodiesterase 4; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

Introduction

Cyclic adenosine 3',5'-monophosphate (cAMP) is the main second messenger of β-adrenergic receptor signalling inducing phosphorylation of L-type Ca²⁺ channels and ryanodine receptor to increase the amount of intracellular Ca²⁺ necessary for heart contractility (Mika et al. 2012; Eschenhagen 2013). And, proper cardiac function relies on fine-tuning balance between the synthesis and degradation of cAMP (Boularan and Gales 2015).

3',5'-cyclic nucleotide phosphodiesterases (PDEs) regulate the localization, duration, and amplitude of the second messengers (Rao and Xi 2009; Mika et al. 2012; Eschenhagen 2013). Of these, phosphodiesterase-4 (PDE4) enzymes with subtypes of PDEA,B,C,D, are crucial in shaping global cAMP signals in cardiac myocytes in rodents (Verde et al. 1999) and in humans (Mika et
Thus, inhibition of PDE4s exhibits hemodynamic and inotropic properties that may be valuable to clinical practice (Goldhaber and Hamilton 2010; Molina et al. 2012; Guglin and Kaufman 2014). Related with this, numerous studies are focused on the mode action of PDE4 inhibitors with their side effects on heart. However, the overall picture of PDE4s inhibition with their inhibitors is still uncertain and additional research is necessary for clinical applicability (Rao and Xi 2009; Mika et al. 2012; Molina et al. 2012; Eschenhagen 2013). To contribute these studies Fourier transform infrared (FT-IR) spectroscopy offers promising approach due to its ability in detection of the changes on content, structure and dynamics of biomolecules in cells and tissues (Derenne et al. 2012; Ozek et al. 2014; Türker-Kaya et al. 2016). It is a rapid technique that does not require any staining nor complicated sample preparation (Derenne et al. 2012; Türker et al. 2014a). Infrared (IR) spectrum of a biological sample represents the sum of various contributions from lipids, proteins, carbohydrates, nucleic acids and all other chemical species (Carmona et al. 2008; Miller et al. 2013; Ozek et al. 2014; Türker et al. 2014a, 2014b). The intensities and/or areas provide quantitative information, peak positions relate to physical states of molecules, and bandwidth gives dynamical information (Severcan et al. 2005; Carmona et al. 2008; Miller et al. 2013; Kumar et al. 2014; Ozek et al. 2014; Türker et al. 2014a, 2014b; Türker-Kaya et al. 2016). As a result, it is possible to monitor global effects of chemicals and conditions on all of the constituents in biological systems (Gasper et al. 2009; Ozek et al. 2014; Türker-Kaya et al. 2016). By taking its advantages FT-IR spectroscopy has been increasingly employed to investigate molecular alterations induced by drugs and even disease’ states, which is not easily detectable by morphological methods, in cells and tissues (Amharref et al. 2006; Akkas et al. 2007; Berger et al. 2010; Leskovjan et al. 2010; Cakmak et al. 2011; Bellisola et al. 2012; Bozkurt et al. 2012; Travo et al. 2012; Türker et al. 2014a, 2014b; Türker-Kaya et al. 2016).

There are different types of PDE4 inhibitors that show distinctive selectivity for PDE4 subtypes. For example, ibudilast has been shown to potently inhibit purified human PDE 4A, 4B, 4C and 4D with IC50 values of 54, 65, 239 and 166 nM, respectively (Huang et al. 2006). Cilomilast is ten-fold more selective for PDE4D compared to other PDE4 subtypes (Rennard et al. 2006). On the other hand, roflumilast does not demonstrate any PDE4 subtype selectivity (Hatzelmann and Schudt 2001). Similarly, as being selective inhibitor of all isoenzymes of PDE4s rolipram, a well-characterized PDE4 inhibitor, was developed as an effective antidepressant, and later tested for treating asthma and chronic pulmonary disease (COPD) (McKenna et al. 2006). Although it was hampered due to its gastrointestinal side effects, it has been still used in research investigating potential use of PDE4 inhibition against several pathologies including cardiovascular diseases (Kenk et al. 2010; Leroy et al. 2011; Molina et al. 2012; Fu 2014).

In the current study, we aimed to acquire a general point of view on the effects of PDE4 inhibition by rolipram at low concentrations (0.05 mg/kg and 1.0 mg/kg) on whole naïve mice heart. We have evaluated the variations of spectral parameters of control and rolipram-treated groups as also performed in previous studies (Cakmak et al. 2011; Bozkurt et al. 2012; Ozek et al. 2014; Türker-Kaya et al. 2016). We have obtained the relative changes in lipid and proteins content, and membrane lipid packing, membrane fluidity, and membrane order and lipid peroxidation, all of which are very important parameters for proper function of heart. Moreover, by detailed analysis of Amide I mode we also predicted the changes in protein secondary structures. We have successfully discriminated control and treated groups depending on their spectra using cluster analysis (Severcan et al. 2010; Türker et al. 2014b). To the best of our knowledge, such approach based on spectral parameters resulted from PDE4 inhibition by rolipram action on naïve heart has been not reported, previously.

Materials and Method

Animal care and drug treatment

Male inbred BALB/c mice (n = 22) weighing 40 g (Mam Tubitak, Gebze, Kocaeli, Turkey), aged 7 weeks upon arrival the laboratory. The animals (4–5 per cage) were kept in the laboratory at 21 ± 1.5°C with 60% relative humidity under a 12 h light/dark cycle (light on at 8.00 p.m.) during 2 weeks before experiments. Tap water and food pellets were available ad libitum. All procedures involving animals were in compliance with the European Community Council Directive of 24 November 1986, and ethical approval was granted by the Kocaeli University Ethics Committee Number: AEK 9/4-2010, Kocaeli, Turkey).

Rolipram were purchased from Sigma Chemical Company (Sigma, St.Louis, MO) and dissolved in saline supplemented with small amounts of dimethyl sulfoxide (DMSO). The drug was freshly prepared before intraperitoneally (i.p.) administration in a volume of 0.1 ml per 10 g body weight. All animals were divided into three groups: control group received vehicle, 0.05 and 0.1 mg/kg doses of rolipram were given to related treated groups for each day during 15 days. At the end of the application procedure the animals were decapitated and whole heart samples were dissected out and kept in –80°C till FT-IR spectroscopic studies.
Sample preparation for FT-IR studies

To eliminate possible biological discrepancy among distinct heart regions, where PDE4 activity is not evenly distributed, we prepared the samples from whole heart tissue. The whole heart samples were dried in a Labconco freeze drier (Labconco FreeZone®, 6 litre Benchtop Freeze Dry System Model 77520) overnight in order to remove the water content. The samples were ground to obtain tissue powder, which were later mixed with dried potassium bromide (KBr) at the ratio of 1/100. The mixture was dried again in the freeze drier for 18 hours to remove all traces of remaining water. And then, in order to obtain same thickness of each pellet same amount of sample was weighted and same pressure ~100 kg/cm² (1300 psi) was applied to produce a thin KBr disk of all samples (Elibol-Can et al. 2011; Turker-Kaya et al. 2016).

FT-IR spectroscopic analysis

Infrared spectra were obtained using a Perkin-Elmer Spectrum One FTIR spectrometer (Perkin-Elmer Inc., Norwalk, CT, USA) equipped with a MIR TGS detector. The spectra of samples were recorded in the 4000–400 cm⁻¹ region at room temperature. Each interferogram was collected with 100 scans at 4 cm⁻¹ resolution. In order to remove the interference of water and carbon dioxide effects on IR spectra the spectrum of air and KBr transparent disk was recorded together as background and subtracted automatically by using Spectrum One software (Perkin-Elmer).

Even though we performed special care to produce same thickness of each KBr pellet, to minimize intra-sample variability which may be caused by variations in experimental conditions, we scanned three independent pellets prepared by taking separate portions randomly from the same sample. The average spectra of three replicates giving identical spectra were normalized and same pressure 100 kg/cm² (1300 psi) was applied to produce a thin KBr disk of all samples (Elibol-Can et al. 2011; Turker-Kaya et al. 2016).

Spectrum One software was used to analyse spectral data by following earlier reports (Elibol-Can et al. 2012; Ozek et al. 2014; Turker-Kaya et al. 2016). The band positions were measured using the frequency corresponding to the centre of 80% × height of the peak. The peak area was calculated as related to a linear baseline between two baseline points which involve the maximum peak height. The baseline point was identified according to start and end points of the peak. Later, the corrected area which is the area between the spectrum and the marked baseline within the marker bar limits was found the peak area value of the interested peak. The bandwidth values of specific bands were calculated as the width at 0.80 × height of the signal in terms of cm⁻¹.

Again still considering possible interference due to detectable variability of sample thickness area ratios of some specific IR modes were also evaluated to approximate concentration changes in biomolecules since we did not apply any biochemical assay to measure concentration of biomolecules of interest as performed previously (Antoine et al. 2010; Turker-Kaya et al. 2016). For the CH₂/lipid, C=O/lipid, olefinic=CH/lipid, and COO⁻/lipid ratios, the areas under the CH₂ asymmetric stretching (2925 cm⁻¹), the C=O stretching (1750 cm⁻¹), the olefinic=CH (3012 cm⁻¹), and the COO⁻ symmetric (1390 cm⁻¹) modes were divided to the total saturated lipid (the sum of the area under the CH₂ asymmetric and symmetric stretching bands). For RNA/protein ratio, the area under C-N-C stretching (998 cm⁻¹) were divided to the sum of the area under the amide I and II bands.

The same software was also used for smoothing, baseline correction and normalization processes. The spectra were first smoothed with nineteen-point Savitsky-Golay smooth function to remove the noise. And then, baseline correction was applied based on specific points. Normalization was performed with respect to specific bands. It should be worth noting that all these procedures were performed only for visual representation of the differences among the groups. For accurate evaluation of the spectral parameters the original average spectrum from each animal was analyzed.

In order to predict the alterations in protein secondary structure elements, amide I mode was further analyzed by OPUS 5.5 (Bruker Optic, GmbH). Since peak height/area of this mode is very sensitive to changes of fullwidth at half height in second derivative spectra, concentration sensitive changes in the components of this band were monitored by measuring intensity values of its sub bands. The spectral comparisons of amide I band were carried out on vector normalized second derivatives.

Measurement of lipid peroxidation (TBARs assay)

TBAR, well-adopted test, was performed to monitor lipid peroxidation (Severcan et al. 2005; Turker et al. 2014a). The heart samples were homogenized by Teflon glass homogenizer in cold 0.02 M phosphate buffer (pH 7.4) at a concentration of 25% (w/v). The homogenates were diluted to 5% with the phosphate buffer containing 0.25 ml buthylated hydroxytoluene (BHT), which prevents artificial increase of malondialdehyde (MDA). Then, the homogenates were incubated for 60 min at 37°C. After the incubation, 2 ml of trichloroacetic acid (TCA) solution (28% w/v in 0.25 N HCl) was added. The samples were centrifuged at low speed and 4 ml of supernatants were mixed with 1 ml of thiobarbituric acid (TBA) (1% w/v in 0.25 N HCl). Subsequently, the samples were kept in boiling water for 30 min to get chromophore development. The samples were cooled to room temperature and the absorbance values were measured at 532 nm.
Hierarchical cluster analysis was performed on first derivative spectra using the cluster analysis module of OPUS 5.5 (Bruker Optic, GmbH). It was applied to distinguish between different spectra from control and treated groups using a frequency range between 4000–800 cm\(^{-1}\). As input data for cluster analysis, spectral distances were calculated between pairs of spectra as Pearson's correlation coefficients (Severcan et al. 2010; Turker et al. 2014b). Cluster analysis for separation of control and rolipram treated heart tissues was based on the Euclidean distances. In all cases, Ward’s algorithm was used for hierarchical clustering.

**Statistical analysis**

The results were expressed as mean ± standard deviation. All data were analyzed statistically using non-parametric ANOVA test. \(p\) value less than or equal to 0.05 was considered as statistically significant.

**Results**

Detailed spectral analysis revealed that there are prominent spectral differences in both rolipram-treated groups compared to control ones. Depending on the spectral variations among groups in the region between 4000–800 cm\(^{-1}\) cluster analyses was performed to differentiate the groups. And, three distinct clusters were produced with a high accuracy (success rate 9/9 for 0.05 mg/kg, and 6/7 tissues for 0.1 mg/kg). The resultant dendrogram is depicted in Figure 1.

In order to easily demonstrate the details of the spectral differences among the groups, the spectra were showed in two separated regions. Figures 2 and 3 show normalized infrared spectra of control, 0.05 mg/kg and 0.1 mg/kg dose of rolipram heart tissues in 3025–2800 cm\(^{-1}\) and 1940–800 cm\(^{-1}\) region, respectively. In Figure 2, the spectra were normalized with respect to the CH\(_2\) asymmetric stretching band at 2925 cm\(^{-1}\), and in Figure 3, the spectra were normalized with respect to the amide I band at 1645 cm\(^{-1}\) for visual demonstration of the spectral variations. The detailed band assignments based upon the literature were given in Table 1. Table 2 represents detailed analysis of spectral modes and results of TBARs assay for each group.

The changes in the content of lipids have been relatively obtained by analysing the areas of the =CH olefinic (unsaturated lipids), the CH\(_2\) asymmetric and the CH\(_2\) symmetric (saturated lipids), the C=O (triacylglycerols, cholesterol esters and phospholipids) and the COO\(^-\) symmetric (fatty acids) as well as olefinic/lipid, CH\(_2\)/lipid, C=O/lipid and COO\(^-\)/lipid ratios. As shown in Figures 2 and 3 and Table 2, in both treated groups all these areas and area ratios were significantly \((p < 0.05)\) reduced.
As demonstrated in Table 2 and Figures 2 and 3, rolipram treatment at both two concentrations led to significant \((p < 0.05)\) shiftings to lower values in the \(\text{CH}_2\) asymmetric stretching \((2925 \text{ cm}^{-1})\), the \(\text{C}=\text{O}\) \((1750 \text{ cm}^{-1})\) and the \(\text{PO}_2^-\) symmetric \((1080 \text{ cm}^{-1})\).

Alterations in fluidity of the cell membrane can be determined by probing the bandwidth values of the \(\text{CH}_2\) asymmetric stretching mode (Lopez et al. 2001; Ozek et al. 2014; Turker et al. 2014b). This value was slightly increased for \(0.05\) mg/kg and for \(0.1\) mg/kg rolipram as illustrated in Figure 2 and Table 2.

The band areas of amide I and amide II modes are directly related with protein content in the system (Elibol-Can et al. 2011; Bozkurt et al. 2012; Turker et al. 2014a). Figure 3 and Table 2 illustrate the areas of these modes significantly \((p < 0.05)\) increased for the group of \(0.05\) mg/kg rolipram but significantly \((p < 0.05)\) decreased for \(0.1\) mg/kg rolipram group.

Amide I mode consists of many overlapping modes that represent different secondary structure elements of proteins such as alpha-helices, beta-turns, turns and random coil (Turker et al. 2014a; Turker-Kaya et al. 2016). Upon analysing of amide I band, the related peaks under amide I were observed in second derivative spectra for control and treated groups (Figure 4). The band at around \(1682 \text{ cm}^{-1}\) is associated with beta turns, the peak at \(1652 \text{ cm}^{-1}\) is due to alpha helix, the mode located at \(1643 \text{ cm}^{-1}\) are assigned to random coil, beta sheet band appears at \(1633 \text{ cm}^{-1}\) (Turker et al. 2014a; Turker-Kaya et al. 2016). The changes in the intensities of characteristics components of amide I mode were given in Table 3. Compared to control for rolipram groups, a decrement in alpha-helix and beta-sheet \((p < 0.05)\) the intensity of beta sheet of amide I band was obtained. More importantly, there is a statistically prominent increment in random coil for both rolipram groups \((p < 0.05)\).

The band centred at \(990 \text{ cm}^{-1}\) is generally assigned to symmetric stretching mode of dianionic phosphate monoester of cellular nucleic acids and ribose-phosphate main chain vibrations of the RNA backbone (Chiriboga

| Frequency (cm\(^{-1}\)) | Definition of the Spectral Assignments |
|-------------------------|----------------------------------------|
| 3012                    | Olefinic =CH stretching: unsaturated lipids |
| 2925                    | \(\text{CH}_2\) asymmetric stretching: mainly lipids with little contribution from proteins, carbohydrates and nucleic acids |
| 2852                    | \(\text{CH}_2\) symmetric stretching: mainly lipid with the little contribution from proteins, carbohydrates and nucleic acids |
| 1750                    | Saturated ester C=O stretch: phospholipids, cholesterol esters, triglycerides |
| 1645                    | Amide I (protein C=O stretch): proteins |
| 1547                    | Amide II (C=N and N-H stretching): proteins |
| 1450                    | \(\text{COO}^-\) symmetric stretching: fatty acids |
| 1080                    | \(\text{PO}_2^-\) symmetric stretching: phospholipids and nucleic acids |
| 976                     | 

Table 1. General band assignment of heart tissue
There was a significant \( \text{p} < 0.05 \) decrease in the area of this band and RNA/protein for both treated groups, indicating a decrease in the nucleic acid, especially RNA content in heart tissue.

**Discussion**

Whole heart tissue contains various components like cardiac myocytes, cardiac fibrocytes, endothelial cells and extracellular matrix. In such complexity FT-IR spectroscopy lacks

**Table 2.** The frequency, band area and area ratio values of FTIR bands and MDA levels for control, 0.05 mg/kg and 0.1 mg/kg rolipram groups of heart tissue

|                  | Control          | 0.05 mg/kg  | 0.1 mg/kg  |
|------------------|------------------|-------------|------------|
| **Frequency**    |                  |             |            |
| \( \text{CH}_2 \) asym | 2924.09 ± 0.07   | 2922.14 ± 0.28* | 2921.88 ± 0.44* |
| \( \text{C=O} \)     | 1753.10 ± 0.89   | 1750.62 ± 1.30* | 1750.09 ± 0.91* |
| \( \text{PO}_2 \) sym | 1080.92 ± 0.58   | 1077.99 ± 1.63* | 1076.81 ± 1.12* |
| **Band area**     |                  |             |            |
| olefinic          | 1.78 ± 0.11      | 1.09 ± 0.07* | 0.34 ± 0.09** |
| \( \text{CH}_2 \) asym | 13.59 ± 0.76     | 12.68 ± 0.14* | 11.27 ± 0.68* |
| \( \text{C=O} \)     | 2.91 ± 0.03      | 1.07 ± 0.08* | 0.90 ± 0.05* |
| amide I           | 25.46 ± 0.78     | 28.51 ± 0.49* | 20.09 ± 1.36* |
| amide II          | 12.27 ± 1.43     | 14.70 ± 1.41* | 10.05 ± 0.83* |
| COO⁻              | 3.90 ± 0.06      | 3.11 ± 0.17* | 3.22 ± 0.34* |
| RNA               | 4.78 ± 0.67      | 3.87 ± 0.39* | 3.79 ± 0.86* |
| **Band area ratio values** |       |             |            |
| olefinic/lipid    | 0.08 ± 0.002     | 0.05 ± 0.009* | 0.02 ± 0.004* |
| \( \text{CH}_2 \)/lipid | 0.61 ± 0.009    | 0.58 ± 0.007* | 0.57 ± 0.006* |
| \( \text{C=O} \)/lipid | 0.13 ± 0.008    | 0.05 ± 0.006* | 0.04 ± 0.001* |
| COO⁻/lipid       | 0.17 ± 0.002     | 0.14 ± 0.002* | 0.15 ± 0.003* |
| RNA/protein      | 0.12 ± 0.007     | 0.09 ± 0.008* | 0.10 ± 0.005* |
| **Bandwidth**    |                  |             |            |
| \( \text{CH}_2 \) asym | 8.44 ± 0.13     | 8.65 ± 0.16  | 8.87 ± 0.95  |
| **TBARS assay**  |                  |             |            |
| MDA (nmol/g)     | 39.44 ± 2.27     | 30.05 ± 1.88* | 31.54 ± 2.90* |

The values are the mean ± SD for each sample. The degree of significance was denoted as * \( \text{p} < 0.05 \).
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Table 3. The changes in value of protein secondary structure estimation by second derivative for control, 0.05 mg/kg and 0.1 mg/kg rolipram groups of heart tissue

| Secondary Structure | Control          | 0.05 mg/kg | 0.1 mg/kg |
|---------------------|------------------|------------|-----------|
| 1-Beta turn         | -0.0022 ± 0.0004 | -0.0019 ± 0.0002\* | -0.0018 ± 0.0003\* |
| 2-Alpha helix       | -0.0049 ± 0.0002 | -0.0045 ± 0.0006\* | -0.0043 ± 0.0007\* |
| 3-Random coil       | -0.0021 ± 0.0005 | -0.0024 ± 0.0009\* | -0.0025 ± 0.0001\* |
| 4-Beta sheet        | -0.0036 ± 0.0007 | -0.0026 ± 0.0004 | -0.0023 ± 0.0006\* |

The values are the mean ± SD for each sample. The degree of significance was denoted as \* p < 0.05.

Lipid molecules are important regulators of cardiac function through their role in membrane phospholipids, as signalling molecules and ligands for nuclear receptors, and as the predominant oxidative substrate for cardiac mitochondria. Regarding this, the effects of PDE inhibitors on such molecules may have prominent importance related to proper heart function. It has been reported that the action of PDE inhibitors increase cAMP level, and thereby raise lipolysis in adipocytes (Chaves et al. 2011). This event may cause deleterious consequences in some pathological conditions such as diabetes mellitus and obesity (Perilli et al. 2013; Arner et al. 2014). Therefore, such inhibitors can be expected to produce some effects on lipid metabolism which could be a potential risk factor for clinical adverse effect in heart. Considering the administration of rolipram to stimulate lipolysis in brown fat tissue (Kraynik et al. 2013) we have approximated the changes in content of lipid components like unsaturated, saturated lipids, cholesterol esters, fatty acids, phospholipids and triacylglycerols. According to the obtained results from the areas of the =CH olefinic, the CH₂ asymmetric, the CH₂ symmetric, the C=O, and the COO⁻ symmetric stretching together with olefinic/lipid, CH₂/lipid, C=O/lipid and COO⁻/lipid ratios, rolipram action has lowering effect on the content of heart lipids. Such decrement in fatty acids, cholesterol esters and triacylglycerols might reveal utilization of these molecules for ATP generation induced by PDE4 inhibition, which might be related with cAMP increasing effect on mitochondrial function and cardiac contractility (Kajimoto et al. 1997; Arner et al. 2014). On the other hand, as another types of lipids, phospholipids have not only pivotal impact on membrane properties but also involve in specific signalling functions necessary for cells to respond to external stimuli. For that reason, membrane phospholipid homeostasis is very important for heart. Related with this, the found decrement in phospholipids together with saturated lipids in the current study has importance for this and shows shortened chain lengths (Turker et al. 2014b). Moreover, such outcome may be reflective of differences in phospholipid signalling that may contribute to the progression of impaired heart function. In sum, it should be noting that the effects of rolipram...
on lipid profile in the blood should be evaluated including levels of cholesterol like low and high density lipoprotein. And, further studies are required to confirm the effects of rolipram on lipid content by measuring lipoprotein lipase activity.

Heart tissue relies on oxidative pathway to utilize fuels, and has high content of mitochondria where reactive oxygen species (ROS) are produced (Armstrong and Ianuzzo 1977; Leary et al. 2003). These molecules readily attack lipids and lipid peroxidation occurs, and LEPs are released into extra and intracellular site of cell (Cakmak et al. 2011; Turker et al. 2014a). Therefore, the detection of LEPs may provide information about not only pathogenesis of any condition but also antioxidant capacity of an agent in tissues including heart, also shown by reports in the literature (Battisti et al. 2008; Ramana et al. 2014). In the current study, we have investigated whether PDE4 inhibition by rolipram stimulates lipid peroxidation in heart, or not. Normally, when lipid peroxidation takes place, an increase in olefinic mode area and olefinic/lipid ratio due to LEPs is observed as reported earlier (Turker et al. 2014a). Moreover, when malondialdehyde (MDA), one of the LEPs, is produced, the increment in the C=O mode and C=O/lipid ratio may be also observed (Cakmak et al. 2011). As shown in Figures 2 and 3 and Table 2, significant decreases in areas of the olefinic and C=O mode and olefinic/lipid and C=O/lipid ratios may show low degree of lipid peroxidation, also confirmed by decreased MDA levels. Here, it should be pointed out that both used concentrations of rolipram (0.05 and 0.1 mg/kg) decreased the content of MDA to approximately the same level. It is very well known that the production of MDA occurs by utilization of unsaturated lipids. As we found that 0.1 mg/kg rolipram has more decreasing effect on the content of unsaturated lipids as obtained from reduced area of olefinic mode at 3012 cm⁻¹. This may result in less MDA level produced than expected. The decrement in LEPs might be resulted from intracellular cAMP level, which can suppress the burst of ROS generation (Azadbar et al. 2009). Hence, this finding which showed decreasing effect of rolipram action on lipid peroxidation level in heart also extends the previous reports performed in different tissues (Rezvanfar et al. 2010; Jindal et al. 2015).

Cardiac action potential is critically dependent on membrane compartments which are proteins and lipids. For that reason, the determination of PDE4 inhibition-stimulated changes on membranes has great importance in understanding the effects of this phenomenon on the proper function of heart. So, we have focused on membrane structure, fully mediated through physical properties of fatty acids, polar head groups of lipids, as well as lipid order, lipid fluidity and content of membrane components However, it should be also mentioning that, our sample contains all membranous structures within heart tissue such as sarcolemma, plasma membrane, sarcoplasmic reticulum, and nuclear membrane. Since our preparation contains all membrane compartments, membrane related parameters might reflect the differences in all membrane systems in heart from control and rolipram groups from a general perspective.

The found decline in the content of phospholipids which also show altered lipid composition further affects the organization and packing of lipids (Marsh 1990; Akkas et al. 2007; Türker et al. 2014b). In order to get information about lipid packing parameter we have analyzed the frequency changes of the CH₂ asymmetric, the C=O and the PO₂ symmetric, which reveal the physical state of fatty acyl chains and head groups of membrane phospholipids. The CH₂ stretching vibrations depend on the degree of conformational disorder; hence they can be used to monitor trans/gauche isomerization in the system (Amharref et al. 2006; Ozek et al. 2014; Türker et al. 2014b). The significant shifting to lower values in the CH₂ asymmetric mode may represent an increment in the order of the system, which presents an increase in the number of trans conformers resulting in more rigid membranes (Curatolo, 1987; Akkas et al. 2007; Elibol-Can et al. 2011; Türker-Kaya et al. 2016). Additionally, significantly lowered wavenumber values of the C=O and the PO₂ symmetric bands may imply an increase in the hydration state of the glycerol backbone near the hydrophilic part and polar head group of the membrane lipids. The hydrogen bonding might be between water molecules and the oxygen molecules of both carboxyl and phosphate groups of phospholipids (Akkas et al. 2007; Carmona et al. 2008; Türker et al. 2014b). The variations in physical state of membrane phospholipids may further affect their interaction with hydrophobic and hydrophilic residues of membrane proteins (Türker et al. 2014b). Furthermore, such outcome may have an effect on the functions of glycolipids and glycoproteins which may operate as cell receptors and be responsible for cell signalling (Saberwall and Nagaraj 1994). All of these structural changes in membranes may alter the activity of membrane proteins.

Lipid organization and composition predominantly determine membrane fluidity, which is important for a number of activities including membrane transport, enzyme activity, chemical secretion, and receptor binding and stimulation (Marsh 1990; Antoine et al. 2010). The found slight increment in the bandwidth value of CH₂ asymmetric stretching mode indicates minor effect of PDE4 inhibition by rolipram on fluidity of heart tissue membranes (Akkas et al. 2007; Elibol-Can et al. 2011; Türker et al. 2014b). This might be resulted from a decrease in low content of cholesterol obtained from lower area of C=O and C=O/lipid ratio.

An interesting finding is that applied concentrations of rolipram are not correlated with the changes in protein content obtained from a significant increase for 0.05 mg/kg but decrease for 0.1 mg/kg rolipram in amide I and amide II mode areas, respectively. This may not be contributed to syn-
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thesis of some proteins since there was a significant decrease in RNA content with increasing concentration (Chiriboga et al. 2000; Banyay et al. 2003; Bozkurt et al. 2012). On the other hand, it has been reported in the literature that increasing concentration rolipram leads to reduction in muscle protein degradation (Lira et al. 2011). An increase in amide I band area for 0.05 mg/kg might be related with this. However, amide I results indicated that besides reducing proteolysis rolipram has also effects on protein profile of whole heart that is non-linearly related with doses. In order to bring conclusion about rolipram action effects on cardiac proteins content the more extensive experimental design studying larger range of concentrations with even isolated proteins would be beneficial.

Since amide I and amide II profiles also depend on the protein structural composition, the changes in areas and amide I/amide II ratio may suggest that there are some alterations in the structures of proteins (Turker et al. 2014b). Moreover, in recent medical research it has been shown that, compared with the normal tissue, the diseased tissue shows a change in protein secondary structure and ratio of amide I to amide II (Szczerbowska-Boruchowska et al. 2007). In order to better estimate the alterations in protein structure amide I mode was further analyzed as performed in earlier studies (Akkas et al. 2007; Turker et al. 2014b; Turker-Kaya et al. 2016). The results of this study showing significant increase in random coils but decrease in alpha helix and beta sheet structures (p < 0.05) might reveal structural change in proteins for rolipram groups. Such changes give information data about protein functions since there is strict relation with their functions and structures. Taking this into consideration, our findings, particularly increase in random coils, reveal denaturation leading to dysfunction of proteins for rolipram groups (Akkas et al. 2007; Turker et al. 2014b; Turker-Kaya et al. 2016). This finding is obviously valuable data since heart muscle contraction is brought about by the interaction of multiple proteins in subcellular structures such as actin, myosin, tropomyosin and troponins. Additionally, again, our preparation contains all membrane compartments, these parameters may be also attributed to the differences in all membrane proteins in heart from control and rolipram groups from a general perspective. For example, a decrease in alpha-helices and beta-sheet structures might suggest that there are changes in membrane lipids between these molecules. This further means that membrane spanning domains of these proteins are then conformationally altered to stabilize membrane curvature (Turker et al. 2014b). All these changes may probably have importance for the regulation of protein functions in heart during PDE4 inhibition. As suggested by Kitsis et al. (1996) such abnormalities in these molecules, major structural and functional components of heart tissue have been postulated to be the cause of contractile dysfunction. These include further cell death and cellular dysfunctions involving contractile proteins, sarcolemma (including associated receptors and channels), sarcoplasmic reticulum and other components of the excitation-contraction coupling apparatus, mitochondria and associated anabolic proteins and various signal transductions. Similarly, it has been reported that unfolded protein response system designed to shut down protein synthesis regulates cardiac sodium current in systolic human heart failure (Gao et al. 2012).

Conclusion

The present study investigated the effects of PDE4 inhibition by rolipram with low two different doses (0.05 and 0.1 mg/kg) in mice heart by FT-IR spectroscopy at molecular level. Detail spectral analysis revealed that rolipram action caused significant changes in the macromolecular content, structure and function in heart tissue. The data indicated differences in membrane packing and a significant decrease in lipid, RNA content, lipid peroxidation but an increase in membrane fluidity and membrane order. Additionally, the change in protein content was found not to be in line with applied concentrations of rolipram. An altered structural profile for proteins was predicted with an increase in random coil whereas a decrease in beta sheet and alpha-helices in both treatment groups.

Depending on these spectral variations control and treated groups could be successfully discriminated by cluster analysis. All these monitored parameters are very important for structure of heart tissue and any change in any of these may further affect its proper functions. For example, alterations in lipids can be observed in myocardial infarction, cardiac arrhythmia and heart failure (Mandel 1995; Moe and Wong 2010; Bui 2011; Harvey and Leinwand 2011; Nordestgaard 2014). Furthermore, the formation of free radicals which further leads to decrease in unsaturated lipids has prominent roles in cardiovascular disease, particularly, atherosclerosis and the associated adverse complications (Smyth et al. 2009; Thomas and Hazen 2010). Moreover, modification in membrane structure in the heart muscle can affect various functions of the membranes by affecting membrane-bound enzymes and receptors directly or indirectly by change in fluidity or permeability of the membranes (Holman 1964; Gudbjarnason and Oskarsdottir 1977). Together with such membrane-related alterations changes in protein structures, it may also cause cardiovascular diseases such as contractile dysfunctions, cardiac amyloidosis, cardiomyopathy and arrhythmia (Mandel 1995; Moe and Wong 2010). For that reason, the findings of the current study can provide a basis for the research on PDE4 inhibition by low doses rolipram, a well-characterized PDE4 inhibitor, and for development of new PDE4 inhibitors.
However, in order to clarify the effects of PDE4 inhibition on heart tissue additional studies including comparison of different PDE4 inhibitors on different animal models of heart failure and arrhythmias should be performed.

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