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ORIGINAL CONTRIBUTION

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Changes in expression levels of genes involved in fatty acid metabolism: upregulation of all three members of the PPAR family (α, γ, δ) and the newly described adiponectin receptor 2, but not adiponectin receptor 1 during neonatal cardiac development of the rat

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Abstract During neonatal cardiac development, the heart changes its substrate preference from glucose to fatty acids. The aim of this study was to investigate the changes in mRNA expression levels of genes involved in the control of cardiac fatty acid metabolism in the transition from neonatal to adult life. *Methods* mRNA expression levels for peroxisome proliferator activated receptor (PPAR) α, γ and δ, PPARγ co-factor 1 α and β (PGC-1 α and β), 9-cis retinoc-acid-activated receptor α, β and γ (RXR α, β, γ), 5'-AMP activated protein kinase (AMPK) α1 and α2, adiponectin receptor 1 and 2 (AR 1 and AR 2) were measured in heart tissue of neonatal 0-day, 7-day and 21-day old rats. *Results* mRNA expression of all three members of the PPAR family were upregulated significantly from day 0 to day 21 (α +117%, γ +133%, δ +203%). In addition, m-RNA expression of all RXR isoforms increased from day 0 to day 7 (α +125%, β +69%; γ +41%). AR 2 exhibited a small but significant increase in mRNA expression (+ 46%). *Conclusions* We were able to demonstrate for the first time that in addition to PPARα, also PPARγ and δ, as well as all RXR isoforms and AR 2 are upregulated in the heart during neonatal development.

Key words Fatty acid metabolism – PPAR – real time PCR – heart – postnatal development

Introduction

In the transition from fetal to neonatal and adult life, cardiac metabolism switches from glucose to fatty acids as a preferred energy substrate to generate ATP [17]. This transition is accompanied by changes in activity and expression levels of several enzymes and regulators involved in fatty acid metabolism. In addition, the adult heart returns to a more "fetal" energy metabolism, i.e., glucose-based metabolism, in certain pathological states like hypertrophy or heart failure [5, 27|. The main regulators of the activity of fatty acid enzymes on the transcriptional level are the so-called peroxisome peroliferator activated receptors (PPARs). PPARs are ligand-activated nuclear receptors which heterodimerize with 9-cis retinoc-acid-activated receptor (RXR) and bind to special PPAR response elements (PPRE) in the promotor region of genes, thereby regulating the transcription of these genes. The role of each individual RXR isoform (α, β, γ) [2] within the PPAR:RXR complex has not been determined yet [3]. However, a tissue specific distribution of RXR isoforms has been described: In rat heart, RXRγ and RXRβ are most abundant, while RXRα is only marginally detected [18].

Natural ligands of PPARs are long chain fatty acids and fatty acid derivates like leukotrienes, prostaglandins and others. Moreover, pharmaceutical agents like lipid-lowering fibrates and insulin-sensitizing thiazolidinediones have been shown to be activating ligands of PPARs [12]. Besides special ligands, there are also transcriptional coactivators like PPARγ coactivator-1 (PGC-

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1) which modulate PPAR activity [20]. So far, three different PPAR isoforms (α, γ and δ) have been described. The distribution of these isoforms varies in different tissues. PPARγ is predominantly expressed in adipose tissue, whereas PPARα and PPARδ prevail in the heart [11]. PPARα and PPARδ play an important role in the regulation of the expression of genes involved in fatty acid oxidation (e.g. medium chain acyl CoA dehydrogynase (MCAD) or long chain acyl CoA dehydrogynase (LCAD)), whereas the role of PPARγ in the heart is not yet clear [15].

A crucial enzyme controlling the balance between glucose and fatty acid consumption by the cardiomyocyte is 5'-AMP activated protein kinase (AMPK) [14]. The enzyme consists of three subunits, the catalytic α-subunit and the non-catalytic β- and γ-subunits. AMPK is activated under conditions such as stress, exercise and fasting, and it facilitates energy producing pathways (like fatty acid oxidation) while inhibiting energy utilizing pathways (like fatty acid synthesis, cholesterol synthesis). Regulation of AMPK activity is complex and involves both phosphorylation and allosteric activation [6].

Furthermore, it has been demonstrated that adiponectin, an adipocyte-derived cytokine, activates AMPK [26, 28]. Adiponectin exerts its effect through recently described adiponectin receptors 1 and 2 (AR 1 and AR 2), which are both expressed in the heart [29]. Adiponectin serves as a link between adipose tissue and muscle fatty acid use [10].

The aim of the present study was to investigate the expression of key metabolic regulators during the maturation of neonatal cardiac metabolism using real time PCR.

Materials and methods

Animals

Myocardial tissue was obtained from Wistar rats raised according to the guidelines for the care and use of laboratory animals by the US National Institute of Health. Animals were sacrificed postnatally at day 0, 7 and 21. Hearts were excised quickly, snap frozen in liquid nitrogen and pulverized in a mortar cooled in liquid nitrogen. Subsequently, myocardial tissue was used for RNA isolation.

RNA purification

RNA was extracted from samples using Trizol reagent (Sigma, Taufkirchen, Germany) according to a standard protocol. The RNA phase was purified using RNeasy Kit colums (Quiagen GmbH, Hilden, Germany) following the protocol supplied by the manufacturer. RNA samples were stored at –80 °C.

RT-PCR and mRNA quantification

Total RNA (1 μg) was transcribed into cDNA using random hexamere primers and Superscript II reverse transcriptase (both Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's protocol in an i-Cycler thermocycler (Bio-Rad Laboratories GmbH, Munich, Germany). mRNA expression was determined using Sybr-green (Eurogentec, Searing, Belgium) on a Taq-

Table 1 Sequences of primers used for real time PCR in this study

Gene	forward Primer	reverse Primer
AR 1	5′-CCACCATGCCATGGAGAAG-3′	5′-ATATTTGGTCTGAGCATGGTCAAG-3′
AR 2	5′-AACAATGACAACCACCACGG-3′	5′-TATCCGCTTTGGAGGATGC-3′
AMPKα1	5′-GCAGAGAGATCCAGAACCTG-3′	5′-CTCCTTTTCGTCCAACCTTCC-3′
AMPKα2	5′-CGGCAAAGTGAAGATTGGAG-3′	5′-CCGTGTTTACAGATGTAGTCGAAC-3′
PPAR α	5′-TGCGGACTACCAGTACTTAG-3′	5′-CGACACTCGATGTTCAGTGC-3′
PPAR γ	5′-CCTGAAGCTCCAAGAATACC-3′	5′-GATGCTTTATCCCCACAGAC-3′
PPAR δ	5′-CTCCTGCTGACTGACAGATG-3′	5′-TCTCCTCCTGTGGCTGTTC-3′
PGC-1α	5′-AACCATGCAAACCACACCC-3′	5′-CATTTGTCTCTGCTGCTGTTTC-3′
PGC-1β	5′-GGAACAGTTATGTGCTGACTTGC-3′	5′-GGAACAGTTATGTGCTGACTTGC-3′
RXR α	5′-CTATCAGCACCCTGAGTTC-3′	5′-TTGAGGACGCCATTGAGG-3′
RXR β	5′-AAGATGTGAAGCCACCAGTC-3′	5′-TCAGGTCCTTCCGAATGGTG-3′
RXR γ	5′-TCAACTTGGTGGTTCCAC-3′	5′-TGGCACAGATGTGTTTCAC-3′
MCAD	5′-GATGATGTGTGCCTACTTGCG-3′	5′-AGGATCTGGGTTAGATCGC-3′
LCAD	5′-TTATCCCCCAGATGACGGC-3′	5′-TCACTCCCAGACCTTTTGGC-3′
18S-rRNA	5′-GGACAGGATTGACAGATTGATAG-3′	5′-CTCGTTCGTTATCGGAATTAAC-3′

Man sequence detector (Applied Biosystems, Foster City, CA, USA). An amount of 0.41 ng of cDNA per reaction was used for determining 18S-rRNA and 12.5 ng for all the other genes. All measurements were performed as duplicates. For each gene, intron-spanning primer pairs (MWG Biotech AG, Ebersberg, Germany) were used in a final concentration of 0.2 μM (see Table 1 for details). Taq-Man cycling conditions were as follows: in an initial step taq-polymerase was activated at a temperature of 95 °C for 15 minutes. Subseqently, amplification cycle conditions were 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s (18S-rRNA) and 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s (for all other genes), respectively. Amplification cycles were repeated 40 times. After each run a melting curve analysis was performed to ensure amplification of correct products. mRNA expression was normalized for levels of cardiac 18S-rRNA as a housekeeping gene, which do not change during neonatal development (Fig. 1A).

Statistics

Results are expressed as mean percent change of mRNA expression ± SD compared to day 0 set at 100 % (n = 6 to 8 rats). Statistical analysis was performed using one way ANOVA. Significant differences were established with a Tukeys test for post hoc comparison, using Prism statistic analysis software (Graph Pad Software Inc., San Diego, CA, USA). A p-value < 0.05 was assumed to be significant, a p-value of < 0.001 was regarded as highly significant.

Results

Expression of all members of the PPAR family increased during neontal cardiac development form day 0 to day 21

As shown in Fig. 1B, mRNA expression levels of PPARα, γ, and δ in the heart all increased markedly and in a highly significant fashion from day 0 to day 7 (PPARα: +109 ± 17 %, $p < 0.001$; PPARγ: +78 ± 29 %, $p < 0.001$; PPARδ: +154 ± 33 %, $p < 0.001$; values represent percent increase compared to day 0 set at 100 %). Only PPARγ and δ mRNA expressions exhibited an even further significant increase from day 7 to day 21 (PPARγ: +55 ± 32 %, $p < 0.05$; PPARδ: +49 ± 24 %, $p < 0.05$). Thus, PPARδ showed the highest increase of all three PPARs. The PPARα and δ target genes MCAD and LCAD also increased significantly but at different rates. Whereas the

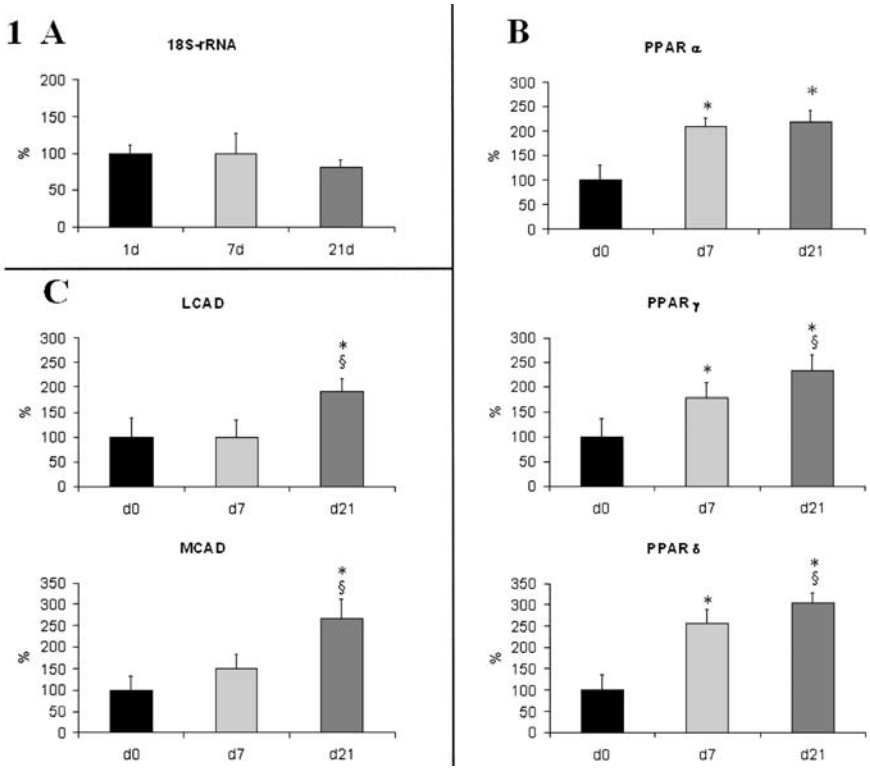

Fig. 1 Cardiac 18S-rRNA levels were determined in rats postnatally at day 0, 7 and 21 **A**. mRNA levels of PPAR isotypes **B** and PPAR target genes LCAD and MCAD **C** were determined in hearts of 0-, 7- and 21-day old rats using real time PCR. Values represent means ± SD (n = 6 to 8 animals/group) expressed in percent compared to day 0 set arbitrarily at 100 %. * significantly different from d 0, § significantly different from d 7, $p<0.05$

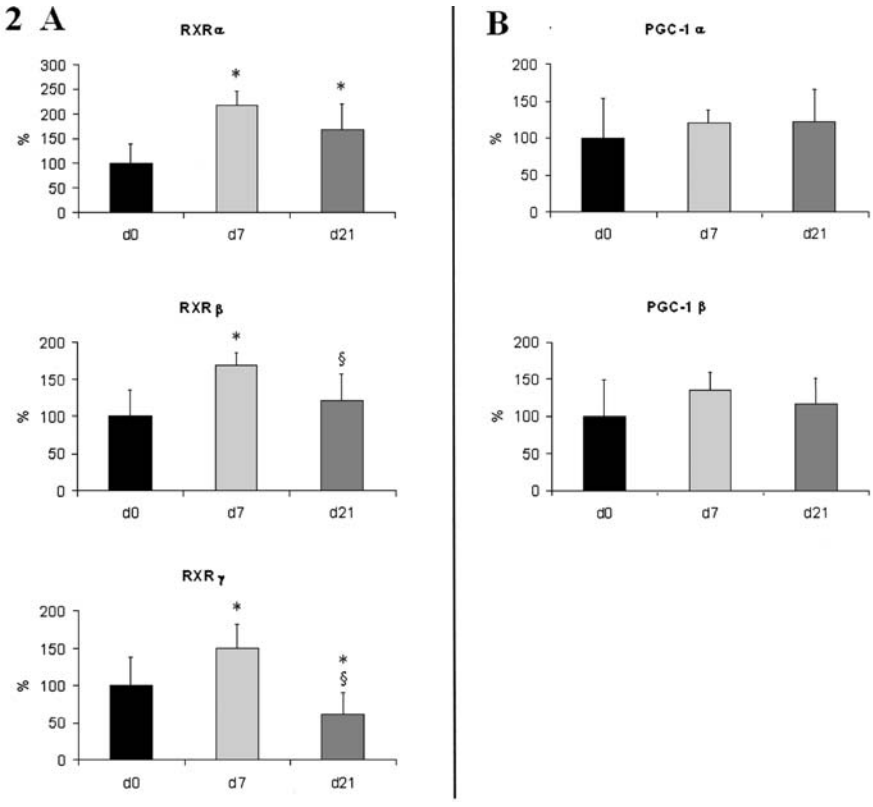

Fig. 2 mRNA levels of PPAR co-factors of the RXR-family **A** and of PGC-1α and β **B** were measured in hearts of 0-, 7- and 21-day old rats. Values represent means ± SD (n = 6 to 8 animals/group) expressed in percent compared to day 0 set arbitrarily at 100%. * significantly different from d 0, § significantly different from d 7, $p < 0.05$

mRNA expression of LCAD did not increase from day 0 to day 7, the concentration of MCAD mRNA increased by 50 ± 34%, but the difference did not reach statistical significance (Fig. 1C). However, at day 21 both MCAD and LCAD mRNA expression increased significantly (MCAD +165 ± 47%, $p < 0.001$; LCAD +110 ± 26%, $p < 0.001$). The mRNA expression of all RXR-isoforms increased significantly from day 0 to 7 (RXRα +117 ± 28%, $p < 0.001$; RXRβ +69 ± 17%, $p < 0.01$; RXRγ +50 ± 32%; $p < 0.05$). Interestingly, the mRNA expression decreased again at day 21: RXRα stayed above and β returned to the level of day 0, whereas RXRγ decreased

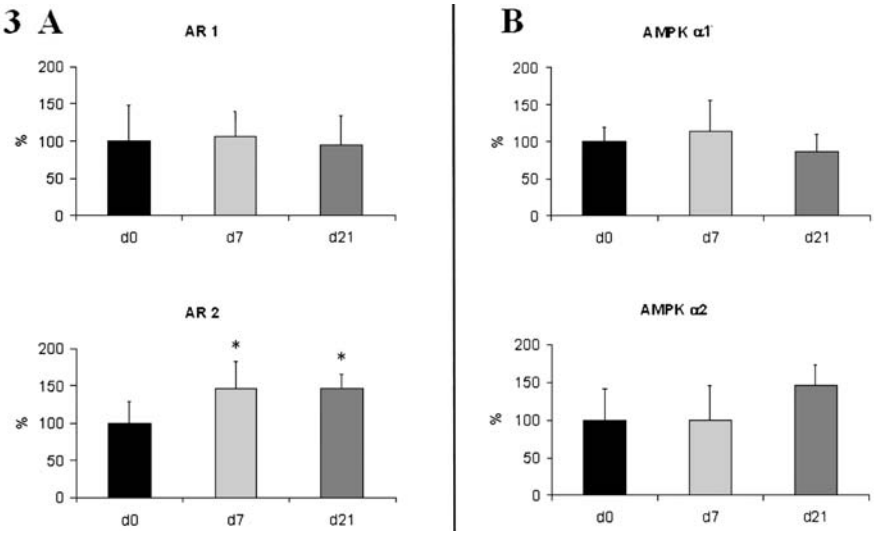

Fig. 3 mRNA levels of AR 1 and 2 **A** and AMPKa1 and a2 subunits **B** were determined in hearts of 0-, 7- and 21-day old rats using real time PCR. Values represent means ± SD (n = 6 to 8 animals/group) expressed in percent compared to d0 set arbitrarily at 100%. * significantly different from d 0, $p < 0.05$

to a level of only 61 ± 28% of the basic value on day 0 ($p < 0.05$) (Fig. 2A). PGC-1 α and β mRNA expressions, both co-activators of PPARα, did not exhibit any changes form day 0 to day 21 (Fig. 2B).

Adiponectin receptor 2 mRNA expression was upregulated in developing rat heart at day 21

AR 2 mRNA expression exhibited a small statistically significant increase of +45% at day 7 and at day 21 (+46 ± 18%, $p < 0.05$), whereas AR 1 expression did not change from day 0 to day 21 (Fig. 3A). No statistically significant changes in mRNA expression levels of the cardiac AMPK a subunits could be detected during neonatal development (Fig. 3B).

Discussion

The present study shows that, during neonatal development of the heart, all three PPAR isoforms are upregulated almost in parallel. To the best of our knowledge, this is the first time that changes in mRNA expression levels of PPARγ and the recently described PPARδ have been studied during neonatal cardiac development.

The simultanous increase in the expression of PPARα and PPARδ is not surprising, since similar functions have been attributed to both nuclear receptors. Cheng et al. [7] demonstrated that both PPARα specific ligand Wy 14643 and PPARδ specific ligand GW0742 induce increased expression of enzymes involved in mitochondrial fatty acid β-oxidation in cell cultures of neonatal rat cardiomyocytes. While the highest increment in mRNA expression of PPARα and δ occurred from day 0 to day 7, the main increment of mRNA expression of MCAD and LCAD occurred delayed from day 7 to day 21 in our study. One possible reason may be the absence of a necessary coactivator, which might be absent during the first seven days of cardiac development. In order to test this hypothesis, we determined the development of mRNA expression of different PPAR coactivators, like the RXR receptors and PGC-1α and β. However, the course of mRNA expression of the examined coactivators does not parallel that of MCAD and LCAD. Thus, the increase in MCAD and LCAD expression cannot be attributed directly to an increased availability of RXR receptors or PGC-1. Interestingly, RXR mRNA levels do increase after birth, even though after three weeks they return to (β) or drop below (γ) the level of expression at day 0. Since we observed an increase in LCAD and MCAD expression at day 21, i.e. after the increase in PPAR and RXR expressions, it is possible that PPAR and RXR in concert trigger further regulatory mechanisms involved in the expression of the fatty acid oxidation enzymes. Nevertheless, further studies will have to be conducted in order to identify potential regulatory pathways of this kind.

In addition, an explanation for the increment in MCAD and LCAD expression could be an increased PPAR activity, which was not determined in the present study. In contrast to PPARα and δ, it is still being debated whether PPARγ plays a role in the heart at all. One reason for doubting a role for PPARγ in the heart is its much lower expression level in comparison to PPARα and δ. Nevertheless, the observed changes in mRNA expression of PPARγ in the present study give rise to the assumption, that like PPARα and PPARδ, also PPARγ has a physiological role in the heart, which increases during neonatal cardiac development. It seems clear that PPARγ has different functions than PPARα and δ. Gilde et al. [13] demonstrated that specific PPARγ ligands do not increase expression of β-oxidation enzymes in cultured neonatal rat cardiomyocytes.

Still, PPARγ may play a protective role in the development of myocardial hypertrophy. Several studies have reported an involvement of PPARγ in the protection from hypertrophy. Asakawa et al. demonstrated that troglitazone, a PPARγ activator, prevented angiotensin II-induced hypertrophy in cultured rat cardiomyocytes [1]. Sakai et al. reported similar results in endothelin 1-induced hypertrophy [22]. The mechanism behind this protective action is not known. NfkappaB seems to be involved in the signaling pathway [25, 30]. Furthermore, a growing number of publications demonstrated an inhibitory effect of PPARγ agonists on the inflammatory response in macrophages [21], during treatment of sepsis [30] or during acute inflammation [9]. In humans with ischemic heart disease, an increase in PPARγ expression has been reported when compared to patients suffering from dilated cardiomyopathy [22]. So far, only few PPARγ specific targets have been identified. The only gene in the heart that exhibits an obvious increase in expression upon treatment with the PPARγ activator troglitazone is acyl CoA oxidase (ACO), an enzyme involved in peroxisomal fatty acid oxidation [4]. In summary, the role of PPAR γ during inflammatory and remodelling processes has to be analyzed in much more detail. It also has to be taken into consideration that the expression levels of PPARs may be different in the main cardiac cell types, i.e. cardiac myocytes, fibroblasts and endothelial cells.

Since maturation of cardiac energy metabolism and its transition from glucose to fatty acid based energy production plays an important role for cardiac function, we studied the time course of mRNA expression of further enzymes involved in metabolism control. A central enzyme in metabolism is AMPK. It has been demonstrated that the protein expression of the AMPKα subunit increases during neonatal cardiac development from day 0 to day 7 in rabbits [16]. In consideration of this data we investigated the development of mRNA expression of

both α subunits of AMPK, but no significant changes of their mRNA levels could be found.

AMPK activity is regulated by a number of different influences (e.g. allosteric activation by increased AMP to ATP ratio, phosphorylation). As mentioned above, adiponectin, an adipose tissue derived hormone, has been shown to lead to AMPK phosphorylation and activation. Recently, the adiponectin receptor, which exists in two isoforms (AR1 and AR2), has been discovered. Both isoforms are expressed in the heart, with the expression of AR1 being higher than that of AR2 [29]. In the present study we demonstrate for the first time a differential expression of the two isoforms of the adiponectin receptor, AR1 and AR2, during neonatal cardiac development of the rat. While AR2 expression increased from day 0 to day 21, AR1 expression remained unchanged. Chinetti et al. [8] described the changes in expression of AR1 and AR2 in human macrophages upon treatment with different PPAR agonists. An activation of PPARα and γ resulted in an increased AR2 expression, while AR1 expression was not affected. Here, we report a parallel increase in mRNA expression of AR2 and all three PPARs, with no change in AR1 expression. We did not measure PPAR activities. However, if we assume an increased PPAR activity due to an increased PPAR expression, our results are in concord with those reported by Chinetti et al. We conclude that an increased PPAR expression during neonatal cardiac development coincides with an increased AR2 expression. Another link between adiponectin receptors and PPARs is the observation by Yamauchi et al. [29] that the overexpression of AR1 and AR2 in C2C12 muscle cells results in an increased PPARα ligand binding and PPAR activity. This would imply that increased adiponectin binding does not only increase fatty acid oxidation via AMPK activation, but also via PPARα activation. In consideration of a number of studies, which postulate a role for AMPK in the regulation of nuclear receptor activities, it is also possible that AMPK itself is involved in activating PPARα. Unfortunately, there is no data available about adiponectin serum levels during postnatal development of the rat. Thus, we could not determine if the observed increase in AR2 expression is a reaction to changes in adiponectin serum levels. In humans, markedly higher adiponectin serum levels have been reported for neonates compared to children and adults [23]. The upregulation of AR2 observed in our sudy could therefore be a reaction to decreasing adiponectin serum levels, representing an adjustment in sensitivity.

In conclusion, in the present study we were able to demonstrate for the first time that not only PPARα, but also PPARγ and δ are upregulated during neonatal cardiac development of the rat. These results are particularly interesting in view of the still not clearly determined role of PPARγ in the heart. In addition, we report that the expression of RXR isoforms ∝, β and γ, important cofactors of PPARs, is increased after birth at day 7, to return to or below perinatal levels at day 21. In concert with PPARs, their physiological partners, they may be part of a regulatory pathway which controls the postnatal increase in expression of fatty acid oxiadation enzymes MCAD and LCAD. Moreover, we could show that AR1 and AR2 are regulated differently during neonatal cardiac development.

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