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Biochemical and morphological effects of K-111, a peroxisome proliferator-activated receptor (PPAR)α activator, in non-human primates

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Dedicated to Prof. Franz Hartig to the occasion of his 75th birthday.

Abstract

K-111 has been characterized as a potent peroxisome proliferator-activated receptor (PPAR)α activator. Antidiabetic potency and amelioration of disturbed lipid metabolism were demonstrated in rodents, which were accompanied by elevations of peroxisomal enzymes and liver weight. To examine the possible therapeutic application of K-111 we have now assessed its efficacy in non-human primates with high transferability to humans. For this purpose obese, hypertriglyceridaemic, hyperinsulinaemic prediabetic rhesus monkeys were dosed sequentially with 0, 1, 3 and 10 mg/kg per day orally over a period of 4 weeks each. In addition, the effect of K-111 on the peroxisome compartment was analyzed in cynomolgus monkeys using liver samples obtained following a 13-week oral toxicity study. In prediabetic monkeys, the reduction of hyperinsulinaemia and improvement of insulin-stimulated glucose uptake rate indicated amelioration of insulin resistance. These effects were nearly maximal at a dose of 3 mg/kg per day, while triglycerides and body weight were lowered significantly in a dose-dependent manner. This reduction of body weight contrasts sharply with the adipogenic response observed with thiazolidinediones, another family of insulin-sensitizing agents. In young cynomolgus monkeys at a dosage of 5 mg/kg per day and more, K-111 induced an up to three-fold increase in lipid β-oxidation enzymes with an 1.5- to 2-fold increase in peroxisome volume density. This moderate increase in peroxisomal activity by K-111 in monkeys is consistent with its role as an PPARα activator and corresponds to the observations with fibrates in other low responder mammalian species. The increase in β-oxidation may explain, at least in part, the lipid modulating effect as well as the antidiabetic potency of K-111. This pharmacological profile makes K-111 a highly promising drug candidate for clinical applications in the treatment of type 2 diabetes, dyslipidaemia, obesity and the metabolic syndrome.

Keywords: Insulin sensitizer; PPAR alpha activator; Non-human primates; Peroxisome proliferation; Type 2 diabetes; K-111 (BM 17.0744)

1. Introduction

Nuclear hormone receptors contribute to the complex control of metabolism by modulating the expression of respective genes. They are transcription factors that bind to the regulatory regions in target genes after activation by specific ligands [1]. This mechanism allows the integration of signals from the environment providing an adequate adaptation of the biological systems [2].

Peroxisome proliferator-activated receptors (PPARs) belong to the family of nuclear hormone receptors that are ligand-activated transcription factors and play a pivotal role in the metabolism of lipids and carbohydrates, energy homeostasis, cell differentiation and proliferation, inflammation, atherosclerosis and cancer [3–6]. Whereas fatty acids and their metabolites are considered to be the natural ligands of PPARs [3,7–9], these receptors are also activated by various classes of drugs such as fibrates, thiazolidinediones and non-steroidal anti-inflammatory drugs [5,8,10–13]. Three subtypes, designated PPARα, PPARβ or PPARδ

Abbreviations: ALAT, alanine aminotransferase; AOX, fatty acid acyl-CoA oxidase; AP, alkaline phosphatase; ASAT, aspartate aminotransferase; PH, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA-dehydrogenase; PPAR, peroxisome proliferator-activated receptor

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and PPARγ, are described in various species ranging from xenopus to humans [14,15]. Each receptor has a distinct species and tissue specific expression pattern [16], with the subtype α being expressed mainly in liver and in adipose tissue, whereas δ is expressed ubiquitously. PPARα is the predominant target for fibrates, a class of drugs well-established in the therapy of dyslipidaemia and hypertriglyceridaemia [17]. Thiazolidinediones bind and activate PPARγ resulting in amelioration of insulin resistance as well as lipid partitioning [8,12,18,19]. Enhancement of reverse cholesterol transport as well as amelioration of dyslipidaemia and hyperinsulinaemia has been described in obese rhesus monkeys after pharmacological activation of PPARδ [15]. A recent study indicates PPARδ as the key metabolic regulator for fat burning [20].

K-111 (formerly BM 17.0744), an ω-substituted alkyl carboxylic acid [21], was characterized recently by Meyer et al. [22] as a potent PPARα activator, without activation of PPARγ. Those findings were confirmed independently by Wurch et al. [23], who in addition found no evidence of PPARδ activation. An antidiabetic potency with insulin-sensitizing and lipid-lowering activities was demonstrated in rodent models of type 2 diabetes [24]. Antihyperglycaemic and antihyperinsulinaemic potencies were stronger than those of troglitazone [25]. The lipid-lowering effects found in metabolically healthy animals also are associated with peroxisomal changes in a species and tissue specific manner [22,25]. In view of the potential therapeutic application of K-111 to type 2 diabetic patients, we have now assessed its efficacy in non-human primates, a species with high transferability to humans [17]. For this purpose obese, dyslipidaemic, hyperinsulinaemic, prediabetic rhesus monkeys (*Macaca mulatta*) [15,17] were used. In addition, the effect of K-111 on the peroxisome compartment in cynomolgus monkeys (*Macaca fascicularis*) was analyzed both biochemically and morphologically using liver samples obtained from a 13-week toxicity study. The results show that K-111 is a potent antidiabetic and hypolipidaemic drug in non-human primates. Only at relatively higher dosages than the therapeutic level, evidence of peroxisomal proliferation is observed with the induction of the lipid β-oxidation enzymes, which is consistent with the role of K-111 as an activator of PPARα.

2. Materials and methods

2.1. Chemicals and drugs

K-111 (2,2-dichloro-12-(4-chlorophenyl)dodecanoic acid) (Fig. 1), was supplied by Fuji Research Labaratories of Kowa Company. All other chemicals, salt mixtures and additives were purchased in the necessary degree of purity from the usual suppliers.

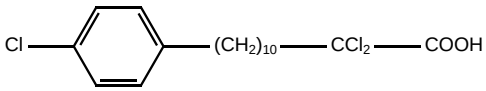

Fig. 1. The chemical structure of K-111.

2.2. Studies with prediabetic rhesus monkeys

A group of six prediabetic obese male rhesus monkeys (11–21 years old) were dosed sequentially with vehicle, 1, 3 and 10 mg/kg per day K-111 for periods of 4 weeks each (Fig. 2). The drug was mixed with jelly and given orally in a piece of banana. Blood sampling for clinical chemistry and euglycaemic hyperinsulinaemic clamp studies were performed at days 14 and 28, respectively, of each treatment period. Peripheral glucose uptake rate during the clamp (M) was corrected for metabolically active mass [26]. Body weight was measured at day 28 of each treatment period. The monkeys were maintained on standard monkey chow (17% kcal protein, 13% kcal fat and 70% kcal carbohydrate; PMI Nutrition International, Inc.) provided ad libitum together with fresh water. Housing and experimental procedures were in accordance with the principles of laboratory animal care of the National Research Council (1996) and approved by the Institutional Animal Care and Use Committee for the University of Maryland. All experimental procedures were carried out after a fasting period of 16 h under light ketamine (Sigma) sedation (10–15 mg/kg body weight). Immunoreactive insulin in plasma was measured using the double antibody method (Linco Research Laboratories). Triglycerides, cholesterol, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and alkaline phosphatase (AP) in plasma were assayed using a Dade Dimension RXL and plasma glucose using a Beckman Autoanalyzer II (Beckman Instruments).

2.3. Studies with normal cynomolgus monkeys

For more detailed investigations of the effects of K-111 blood and liver samples from cynomolgus monkeys (4–6 years old from China National Scientific Instruments & Materials Import/Export Corporation) of a 13-week oral toxicity study were used. Male and female monkeys, 32 in total, were divided into the main study and the recovery groups. The main study groups consisted of six animals (three males and three females per group) each treated with vehicle (control), 5, 10 or 20 mg/kg per day K-111. The recovery groups consisted of control and 20 mg/kg per day groups with four monkeys each (two males and two females per group), which were kept and observed for 4 more weeks after the end of the dosing period (Table 1). The animals were housed individually and fed 108 g of solid food daily (Teklad Certified 25% Monkey Diet (W), Harlan Sprague–Dawley Inc.). Food intake was exactly monitored by weighing the uneaten food the following day. Water was available ad libitum. Methylcellulose (metrose SM-400, Shin-Etsu Chemical Co., Ltd.) solution (0.5%, w/v) was used for vehicle at a dose volume of 2.5 ml/kg.

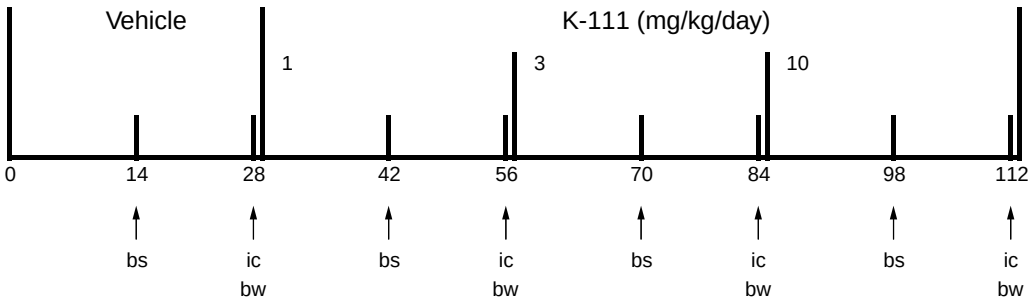

Fig. 2. Study design in prediabetic obese male rhesus monkeys: six prediabetic rhesus monkeys were treated sequentially with vehicle 1, 3 and 10 mg/kg per day K-111. Blood sampling (bs), glucose clamp (ic), body weight (bw) measurement were carried out as indicated.

Animals were administered the vehicle or the drug via a nasogastric catheter. Glucose, triglycerides, cholesterol, ASAT, ALAT and AP in plasma were assayed using an autoanalyzer (Clinicalyzer RX-10, JEOL, Ltd.).

One day prior to necropsy of the cynomolgus monkeys the food was removed at approximately 5:00 p.m. The animals were euthanized by exsanguination under anaesthesia with intravenous injection of sodium pentobarbital (64.8 mg/ml, 0.4 ml/kg) into the cephalic vein.

To determine fatty acid acyl-CoA oxidase activity (AOX) [27], protein and lipid content, pieces of the right lobe of liver (ca. 2 g) were perfused with saline, frozen in liquid nitrogen and stored at −80 °C. The measurements were performed using a spectrophotometer U-3200, Hitachi Co., Ltd.

For analysing several peroxisomal proteins and PPARα, a portion of liver (left lobe, approximately 5 g) was collected from all animals. The sample was washed with saline, weighed and frozen immediately with liquid nitrogen and stored in a deep freezer (−80 °C).

2.4. Tissue preparation

After exsanguination the liver was immediately washed with physiological saline. For immunoblotting, a portion of the liver was frozen with liquid nitrogen and stored in the deep freezer (−80 °C). For immunocytochemistry, 1 mm × 4 mm pieces of the liver were collected and immediately fixed for a total of 60 min in the following fixative: 4% (w/v) depolymerized paraformaldehyde, 0.05% (v/v) glutaraldehyde, 0.05% (w/v) calcium chloride, 2% (w/v) sucrose, 0.1 M Pipes buffer (pH 7.4). After fixation the liver portions were put in 0.15 M Pipes buffer (pH 7.4) containing 2% (w/v) sucrose at 4 °C.

2.5. Preparation of antibodies

Monospecific polyclonal antibodies against catalase, AOX, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA-dehydrogenase (PH) and PPARα protein were raised in rabbits as described previously. The specifity of the antibodies was assessed by immunoblotting [28]. The antibodies (0.01 μg IgG/μl) were diluted before use for immunocytochemistry: catalase (1:2000) and for immunoblotting: catalase (1:2000), PPARα (1:1000), AOX (1:300), PH (1:300).

2.6. Immunoblotting

Hundred to four hundred milligrams of the frozen liver tissue were suspended in TVBE-buffer (1 mM $NaHCO_3$, 1 mM EDTA, 0.1% (v/v) ethanol, 2% (v/v) Triton X-100) and homogenized with a Potter S Homogenizer (Braun Melsungen) with 1500 rpm (1 min on ice). For detection of PPARα, the liver samples were suspended in a special buffer (10 mM Tris–HCl, 20 mM Na-molybdate, 1.5 mM EDTA, 0.6 M KCl, 3 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 M benzamidine, 1 M 6-amino-capronic acid) homogenized and centrifuged at 100,000 × g (60 min at 4 °C) using an ultracentrifuge (Beckmann L5-65B, Beckman Instruments). The protein content of the homogenates was determined with the Bradford method.

Table 1
Study design with healthy cynomolgous monkeys

Group	Test article	Dose level	Number of animals[a]	
			Male	Female
Control	0.5% Methylcellulose	0 mg/kg per day	3 + 2[b] (1–5)	3 + 2[b] (6–10)
5 mg/kg per day	K-111	5 mg/kg per day	3 (11–13)	3 (14–16)
l0 mg/kg per day	K-111	l0 mg/kg per day	3 (17–19)	3 (20–22)
20 mg/kg per day	K-111	20 mg/kg per day	3 + 2[b] (23–27)	3 + 2 (28–32)

[a] The numbers in parentheses are the individual animal numbers.
[b] Recovery animals (1, 2, 6, 7, 23, 24, 28, 32).

For immunoblotting equal amounts of heat-denatured proteins of treated and control animals were subjected to Ready Gels (Bio Rad: 12% resolving gel, 4% stacking gel) and electrophoresed [29]. The appropriate amount of protein for each sample was determined based on the affinity of the corresponding antibody (catalase: 0.5; AOX 1.5; PH 10 and PPARα 10 μg protein per lane). Resolved polypeptides were electrotransferred onto polyvinylidene difluoride (PVDF) membranes [30] with the semi-dry system [31]. After blotting the membranes were incubated for 30 min with 0.3% (v/v) milk in phosphate-buffered saline (PBS)/Tween to block unspecific binding sites. Incubation with primary monospecific antibodies was performed at room temperature over night in 1% (v/v) fetal calf serum in the same buffer. After repeated washing, a peroxidase-conjugated goat anti-rabbit antibody (1:20,000) was added for 1 h at room temperature and immune complexes were visualized by enhanced chemiluminescence (NEN). The immunoblots were quantitated using a digital image station (Image Station 440 CF, Kodak Digital Science, NEN).

2.7. Immunocytochemistry

The liver specimens for immunocytochemistry were embedded in LR-White (Sigma) [32] and cut into 1 μm sections with an ultramicrotom (Reichert-Jung Ultracut S, Leica). The sections were put on special anti-frost slides and processed for immunocytochemical localization of peroxisomal catalase. The sections were treated for 30 min with TNB (0.1 M Tris–HCl, 0.5% (v/v) blocking reagent (NEN), NaCl-buffer, pH 7.5) to block unspecific binding sites, and incubated overnight with the primary antibody (rabbit anti-catalase-IgG) in PBS. After washing (0.2% (w/v) BSA-C in PBS) the sections were incubated for 60 min with gold-anti-rabbit-IgG (Sigma) diluted 1:100 in 0.2% (w/v) BSA-C/PBS. Subsequent post-fixation with 2% (v/v) glutaraldehyde/PBS and washing with distilled water was followed by contrasting with Bio Cell Silver Enhancer Kit (British Bio Cell International).

2.8. Morphometry

After immunocytochemical localization of the peroxisomal catalase the volume density of peroxisomes in tissue sections was determined using a television-based image analysis system (Quantimed 500, Leica), with an oil-immersion objective (40×) and an Orthoplan light microscope (Leica), as described previously [33].

2.9. Statistical analysis

The two-tailed *t*-test was used to evaluate differences compared to vehicle-treated controls. Differences among means were considered statistically significant at $P < 0.05$, and statistically highly significant at $P < 0.001$.

3. Results

3.1. Reduction of body weight, increase in glucose uptake and amelioration of hyperinsulinaemia and hyperlipidaemia in prediabetic obese rhesus monkeys by K-111

Significant individual differences were found for plasma insulin (Fig. 3a), glucose uptake rates (Fig. 3b), triglycerides (Fig. 3c) and body weight (Fig. 3d) at the end of the vehicle treatment period (baseline). Individual changes of these parameters for each monkey at each drug treatment dose level relative to baseline are given, therefore, together with the absolute baseline values (top line of each figure).

Reduction of hyperinsulinaemia to varying degrees was found in all treated monkeys, although the level of decrease did not correlate with the extent of hyperinsulinaemia. Most of the effect was observed already with the lowest dose of 1 mg/kg per day K-111 in four out of six animals. Glucose uptake rate was enhanced significantly in all monkeys with the 3 mg/kg per day dose of the drug and the further increase in dosage did not amplify this effect. The extent of absolute triglyceride lowering effect was the lowest in animals exhibiting pretreatment values below 200 mg/100 ml. The highest effect, a reduction of 275 mg/100 ml, was found with a dosage of 10 mg/kg per day in the animal (A), that started with severe hypertriglyceridaemia at baseline (346 mg/100 ml). The total serum cholesterol was not altered by drug treatment (data not shown). Body weight was reduced in all monkeys beginning with the lowest dose of 1 mg/kg per day K-111 in a dose-dependent manner. Thus, it seems that the antidiabetic, lipid lowering and weight reducing effects are all part of the pharmacological profile of K-111, which was well-tolerated in all dosages given. There were no signs of toxicity with regard to the behaviour of the monkeys or the clinical chemistry. A more detailed report of the hormonal status with clinical chemistry and haematology data of the same animals is presented elsewhere [34].

3.2. Reduction of body weight and enhancement of peroxisomal β-oxidation enzymes in healthy cynomolgus monkeys by K-111

3.2.1. Body weight

The body weight in male and female cynomolgus monkeys, which were mostly lean young adult animals, was slightly increased in the control groups (receiving vehicle) at the end of the treatment period, in male animals by 4.83% and in female animals by 5.12%. In contrast, animals receiving 20 mg/kg per day of K-111 for 13 weeks showed a significant reduction of body weight with a decrease of 13.13% in males and 6.67% in females. In the lower dose groups the effects on body weight were somewhat less pronounced in these lean animals. Because of the observed body weight reducing effects of K-111 the

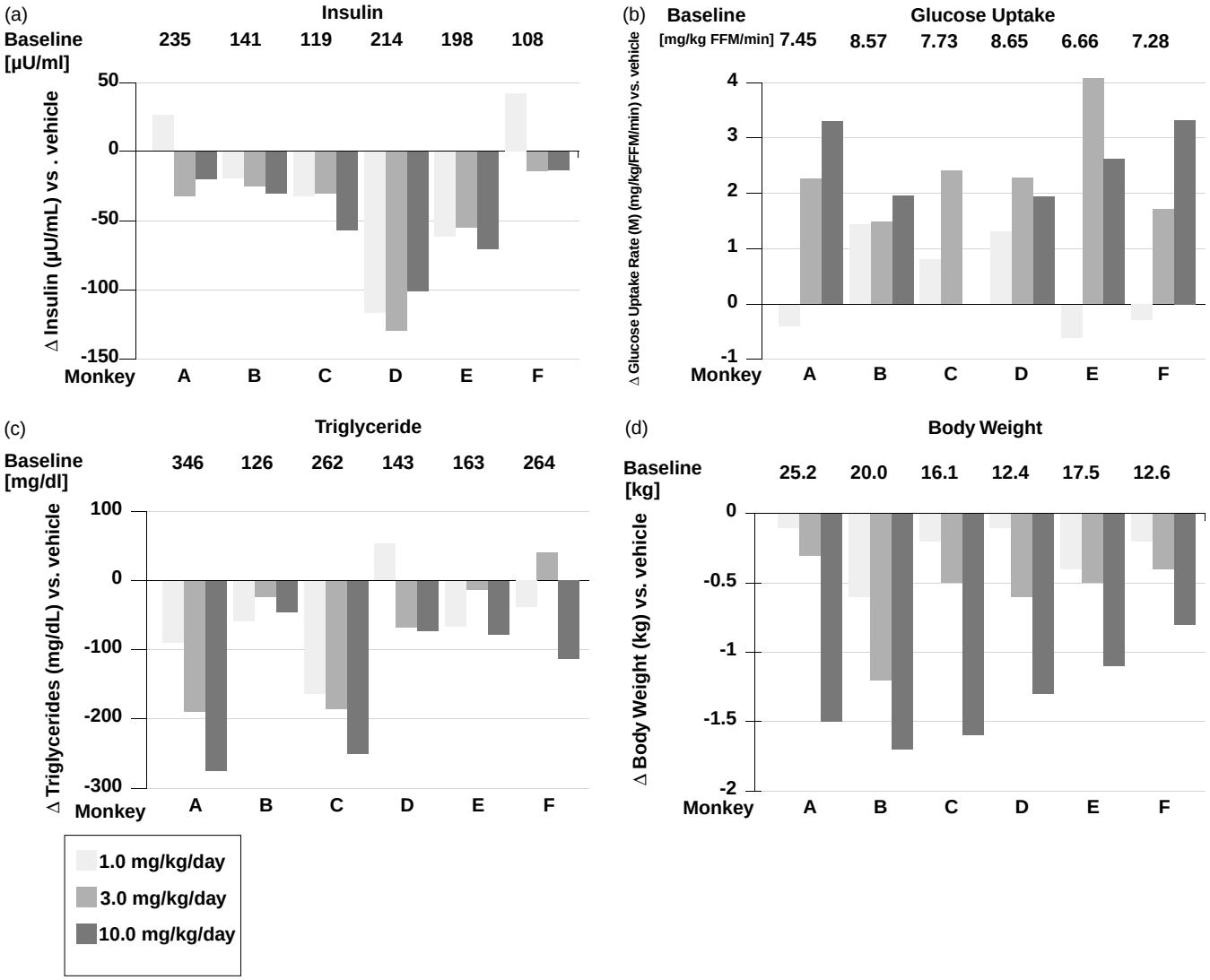

Fig. 3. Six prediabetic obese male rhesus monkeys were dosed sequentially for 4 weeks each with vehicle (baseline), 1, 3 and 10 mg/kg per day K-111. Blood sampling for insulin and triglycerides was performed at day 14 and glucose clamp studies and body weight measurements were carried out on the 28th day of each treatment period. Individual alterations in each drug treatment period relative to baseline are given for plasma insulin (a), glucose uptake rate during a euglycaemic hyperinsulinaemic clamp (b), plasma triglycerides (c) and body weight (d). The absolute baseline values for each parameter are given on the top of each figure. The letters below each figure refer to the individual animals.

relative liver weight is presented both in relation to brain (Fig. 4) as well as to the body weight (Table 2).

3.2.2. Serum lipids, glucose and enzymes

Cholesterol, triglycerides and glucose as well as ASAT, ALAT and AP in blood did not show any time or dose related changes in monkeys for either sex (data not shown).

3.2.3. Liver weight

The absolute liver weights (Table 2) in males were slightly higher in two out of three of the low and medium dose groups and in all animals of the high dose group at the end of the drug treatment. Considering that in females the highest liver weights were found in the recovery control group (98 g) and in the high dosed group (98.5 g), it seems that the effect is rather minimal. Because of the reduction of body weight, the relative liver weight as related to body weight appeared increased dose-dependently in most animals (Table 2). Such a dose-dependent elevation of liver weight was not observed when the liver weight was related to the brain weight (Fig. 4). Slightly higher values of 1.76 and above were obtained in one of the middle (animal no. 18) and two of the high dose group (animal nos. 25 and 27) of males and one of the high dose group (animal no. 30) of females only.

3.2.4. Acyl-CoA oxidase (AOX) activity in liver

The activity of AOX in liver showed marked individual variation in controls and was increased in a dose-dependent manner in treated animals with a reduction of enzyme activity after the recovery period (Fig. 5). The elevations were more pronounced in females with one animal

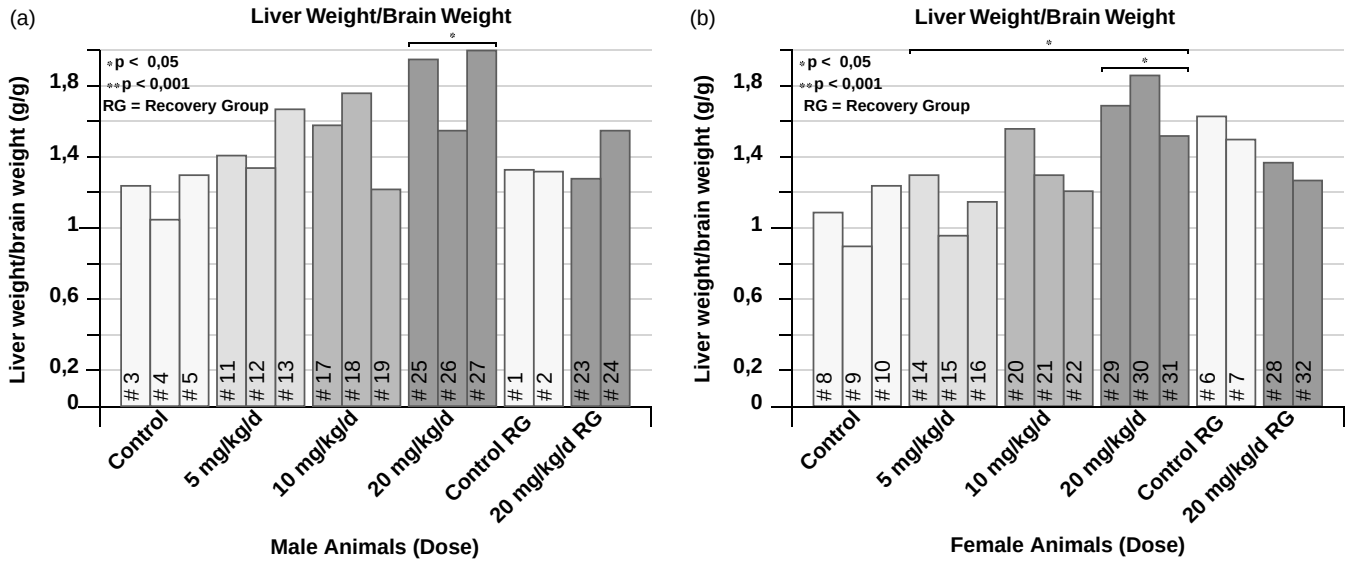

Fig. 4. Liver weight/brain weight ratio in cynomolgus monkeys of both sexes in the different dosage groups after oral administration of K-111 or vehicle (methylcellulose) over 13 weeks: (a) males and (b) females. The recovery period lasted 4 weeks. The numbers in each column refer to individual animals.

showing about three-fold increase after treatment with the highest dose (animal no. 31).

3.3. Immunoblotting

3.3.1. A dose-dependent elevation of the protein levels of β-oxidation enzymes, AOX and PH in K-111-treated animal groups

3.3.1.1. Acyl-CoA oxidase (AOX). In male animals (Fig. 6a) a significant increase of AOX protein content in all groups was observed: in the 5 mg/kg per day group a rise of ca. 100%, in the 10 mg/kg per day group an increase of about 150% and in the 20 mg/kg per day group an increase of about 200%. The male 20 mg/kg per day recovery group showed only a slight decrease of AOX-protein content relative to the 20 mg/kg per day non-recovery group remaining still highly significantly elevated above both control groups. All treated female animals (Fig. 6b) also showed a significant rise of AOX-protein relative to control groups. In the 5 mg/kg per day and the 10 mg/kg per day groups comparable values were

Table 2
Liver weight in control and treated male and female cynomolgus monkeys given in absolute and relative values

Study groups	Male animals			Female animals		
	Animal numbers	Liver weight (g)	Liver weight per body weight (g/kg)	Animal numbers	Liver weight (g)	Liver weight per body weight (g/kg)
Control	3	86.3	16.99	8	65.6	20.44
	4	83.5	19.28	9	61.2	19.74
	5	95.7	16.79	10	66.9	19.68
5 mg/kg per day	11	105.2	21.51	14	79.8	25.02
	12	85.3	20.02	15	69.0	21.56
	13	111.2	21.59	16	69.1	22.81
10 mg/kg per day	17	118.2	23.41	20	79.1	24.26
	18	106.6	22.35	21	86.8	24.94
	19	77.9	19.82	22	70.9	24.36
20 mg/kg per day	25	107.6	28.47	29	97.4	30.15
	26	108.7	30.03	30	98.5	30.50
	27	124.7	25.87	31	90.0	30.93
Control RG[a]	1	90.6	18.72	6	98.0	24.02
	2	87.6	20.52	7	89.4	25.99
20 mg/kg per day RG[a]	23	93.3	20.83	28	71.8	22.44
	24	105.8	26.78	32	73.9	24.97

[a] RG: recovery group.

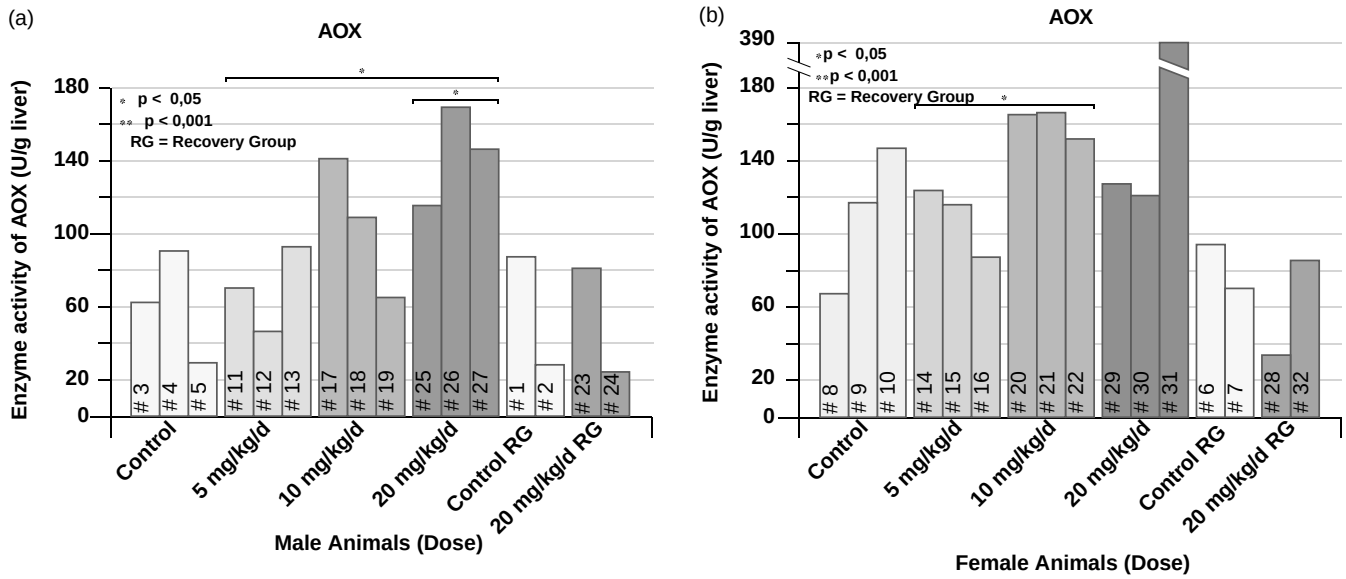

Fig. 5. Activity of fatty acyl-CoA oxidase in livers of male (a) and female (b) cynomolgus monkeys, in treated and in control animals (for details see Fig. 4). Note the marked increase of AOX activity in a dose-dependent manner and a reduction of enzyme activity after the recovery period.

found, with an increase of about 78% and further elevation of 200% in the 20 mg/kg per day group. Although the 20 mg/kg per day recovery group showed a slight decrease of AOX-protein content relative to the 20 mg/kg per day non-recovery group it still remained significantly elevated. Individual differences within various groups could be observed in both male and female animals.

3.3.1.2. Peroxisomal hydratase-dehydrogenase (multifunctional protein) (PH). In male animals (Fig. 6c) there was a significant increase in the PH protein content in all treated groups. The 5 mg/kg per day as well as the 10 mg/kg per day group showed an increase of about 300% and the 20 mg/kg per day group showed an increase of ca. 500% compared to the control group. The male 20 mg/kg per day recovery group showed a slight decrease of the PH protein content but remained above both control groups and above the two lower dose groups. In female animals (Fig. 6d) the 5 mg/kg per day group showed an increase of about 150%, the 10 mg/kg per day group a rise of ca. 250% and the 20 mg/kg per day group an increase of about 300%. The 20 mg/kg per day recovery group showed no significant protein reduction compared to the 20 mg/kg per day group of the main study without a recovery period. Individual differences within groups were observed especially in the male animals.

3.3.1.3. Peroxisome proliferator-activated receptor α (PPARα). The analysis of PPARα protein (Fig. 6e and f) revealed marked variations in individual male and female animals with no significant alterations due to treatment in the different dosing groups.

3.3.1.4. Catalase. The amount of catalase protein did not show any significant alterations in the different dosing groups, either in male or in female animals (data not shown).

3.4. Immunohistochemistry

No significant pathologic alterations were found in liver sections stained with haematoxylin–eosin. By immunohistochemistry fine dark granules in cytoplasm of hepatocytes corresponding to the distribution of peroxisomes were clearly visualized in the livers of cynomolgus monkeys and a significant peroxisome proliferation was observed in treated animals (Figs. 7 and 8). The morphometric analysis of peroxisomal volume density by automatic image analysis (Figs. 7g and 8g) revealed an increase to about 1.5- to 2-fold of the control values in a dose-dependent manner. The peroxisomal volume density was reduced in the 20 mg/kg per day recovery group, this reduction being statistically significant in the female animals. Nevertheless, the values of the recovery groups still remained significantly higher than those of both control groups.

4. Discussion

4.1. Pharmacological profile of K-111 in non-human primates

In view of the pleiotropic nature and the complex pathophysiology underlying type 2 diabetes and its late complications, drugs that intervene at multiple steps of the disturbed metabolism should be better suited for therapeutic interventions [35,36]. Today, this is achieved by combining different agents with divergent modes of action [37–39]. Interestingly, PPARs play a crucial role in the regulation of the metabolism as well as in the inflammation

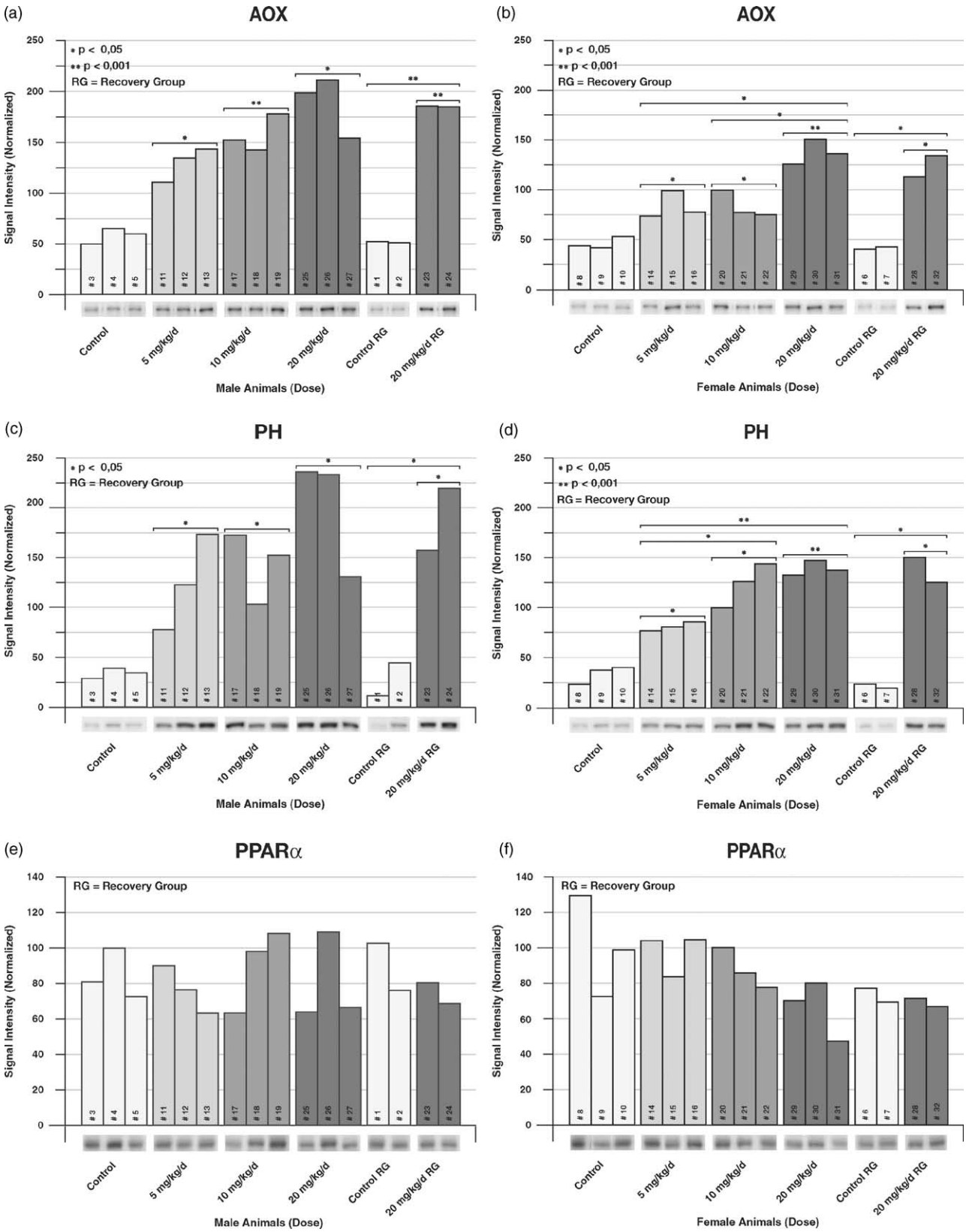

Fig. 6. Immunoblots of liver homogenates from cynomolgus monkeys of both sexes (left panels: males, right panels: females, for more details see Fig. 4). The same quantity of protein was applied from controls and treated animals in each case. The immunoblots were quantified using a Kodak digital imaging station. All values given are normalized using animal # 20 as reference (100%). Determined proteins: (a and b) AOX (acyl-CoA oxidase); (c and d) multifunctional protein PH (enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase); (e and f) peroxisome proliferator-activated receptor α (PPARα). Note the dose-dependent significant increase in the amount of PH and AOX-proteins. No significant alteration could be observed for PPARα in the treated animal groups.

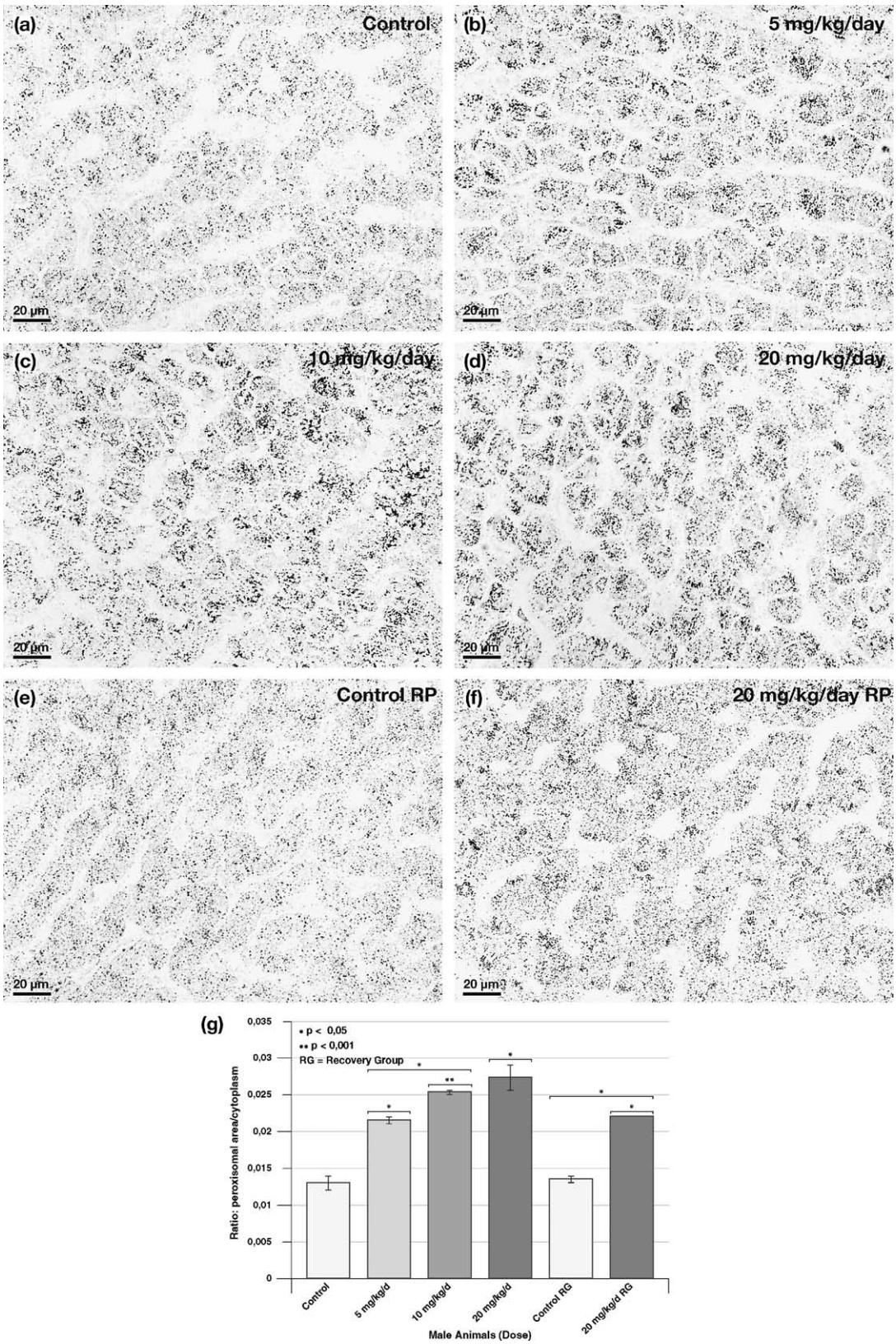

Fig. 7. (a–f) Light micrographs from the livers of male cynomolgus monkeys (for details see Fig. 4) after immunocytochemical visualization of peroxisomes with an antibody to catalase and gold-labelled-IgG: (a) control animal; (b–d) treated animals (dosage as indicated); (e and f) control and treated animals after the recovery period (RP) and (g) volume density of peroxisomes determined by automatic image analysis of the immunocytochemically processed sections. The values present the volume fraction of peroxisomes as a percentage of the hepatocyte volume. Note the dose-dependent augmentation by 1.5- to 2-fold in treated animal groups and a slight reduction of the peroxisomal volume density in the treated group after the recovery period.

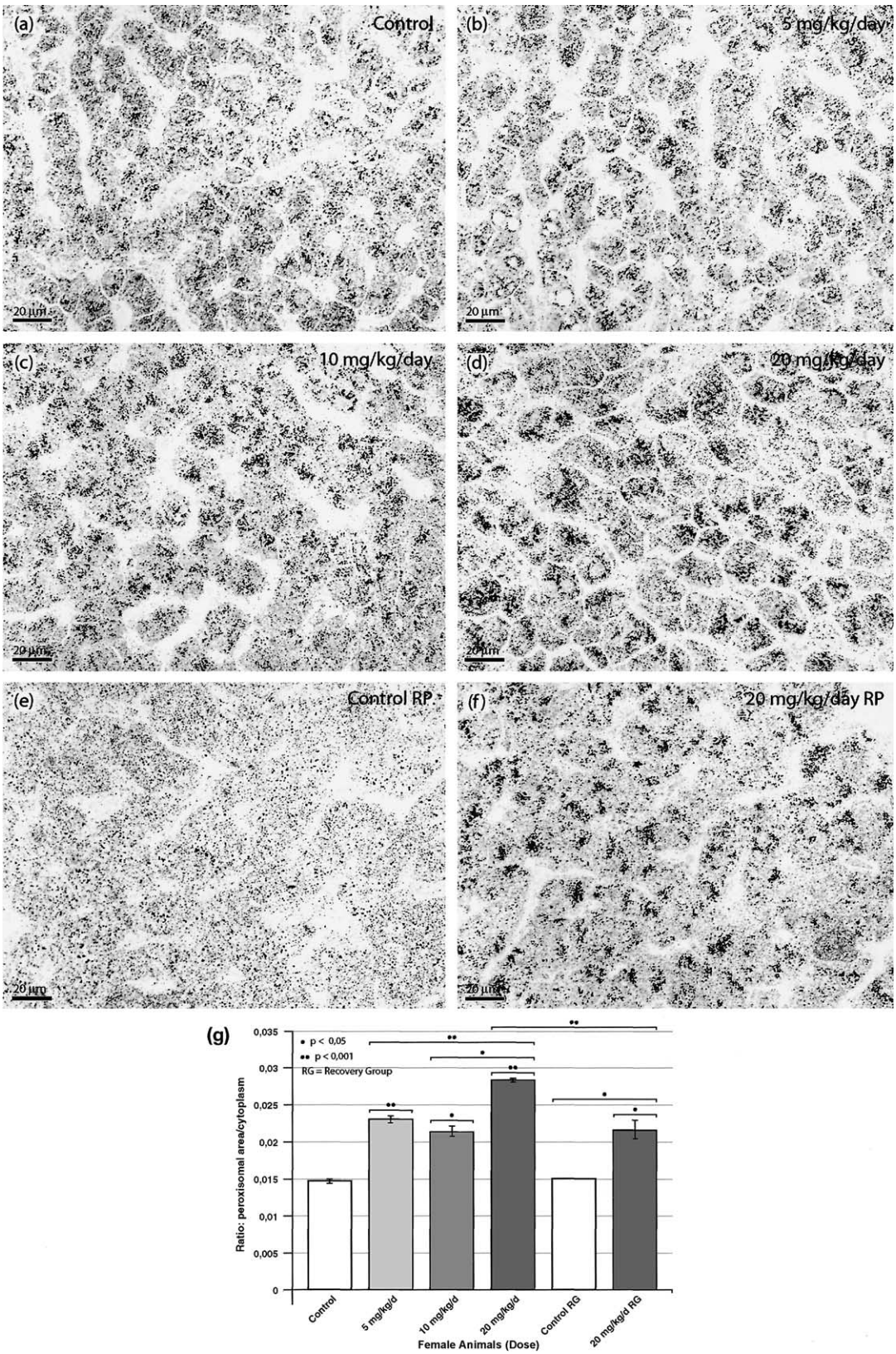

Fig. 8. (a–f) Light micrographs from the livers of female cynomolgus monkeys (for details see Fig. 4) after immunocytochemical visualization of peroxisomes with an antibody to catalase and gold-labelled-IgG: (a) control animal; (b–d) treated animals (dosage as indicated); (e and f) control and treated animals after the recovery period (RP) and (g) volume density of peroxisomes determined by automatic image analysis of the immunocytochemically processed sections. The values present the volume fraction of peroxisomes as a percentage of the hepatocyte volume. Note the increase by 1.5- to 2-fold in treated animal groups and the reduction of the peroxisomal volume density in the treated group after the recovery period.

contributing to the late diabetic complications of the vascular system [2–6,20]. K-111 has been characterized as a potent PPARα activator with strong lipid-lowering and antidiabetic response in rodents [22–24]. The investigations in obese, hypertriglyceridaemic, hyperinsulinaemic prediabetic rhesus monkeys now have extended those observations to non-human primates, a species with high transferability to humans [17].

Insulin resistance is a primary feature in the onset and progression of type 2 diabetes [40–42]. In obese rhesus monkeys K-111 reduced hyperinsulinaemia without changing the blood glucose suggesting a reduction of insulin resistance. This conclusion is also supported by the amelioration of impairment of glucose uptake. A significant lowering of triglycerides, with strongest effects in monkeys with the highest baseline values (Fig. 3), is in accordance with the regulatory function of PPARα on lipid metabolism [10,14] and may explain the absence of lipid-lowering effect in normolipaemic cynomolgus monkeys.

The prediabetic rhesus monkeys differ in their genetic background as well as pathophysiology which is clearly demonstrated by differences in baseline values. The almost uniform pharmacological responses observed may be an indication that the action of K-111 is independent of the nature of the metabolic disturbance, which is in agreement with findings in rodents [24]. The absence of hypoglycaemia in both monkey studies as well as in our previous report on rodents [24] confirms that K-111 should have no risk of hypoglycaemia in humans.

Obesity and other forms of disturbed lipid homeostasis are highly correlated with cardiovascular diseases as well as the onset and progression of type 2 diabetes [43–45]. The induction of β-oxidation enzymes and enhanced expression of uncoupling protein (UCP) by specific ligands of PPARα have been reported [46]. Those findings could explain the reduction of body weight observed in obese, diabetic rhesus monkeys as well as in metabolically normal cynomolgus monkeys. The lipid β-oxidation and enhanced UCP expression contribute to energy dissipation and finally reduction in lipid storage and body weight. The proposed mechanism of weight reduction by K-111 is in contrast to that of the thiazolidinediones which induce elevation of body weight [12,19]. Reduction of body weight by K-111 is a highly interesting aspect of its pharmacological profile, because presently obesity is a major health problem in industrialized countries [47,48]. PPARα has recently been identified as a modulator of body weight [49]. Further studies on the exact mechanism of action of K-111 on obesity and body weight are warranted.

4.2. Differences in response to peroxisome proliferators between rodents and primates

Species differences in response to treatment with peroxisome proliferators are well-known [50–53]. The so-called high responders, such as rats or mice, show, depending on the type of compound used, dosage and treatment time, 50- to 100-fold increase in lipid β-oxidation activity and up to 10-fold augmentation of the volume density of peroxisomes with marked hepatomegaly and tumorigenesis. In contrast, low- or non-responders, such as guinea pigs, monkeys or humans, only exhibit slight peroxisome proliferation (1.5- to 2-fold) with moderate (2- to 3-fold) induction of peroxisomal enzymes and no pathological alterations or tumors [50–52]. Fahimi et al. [50] compared the responses of rats and monkeys to the same dosage of 75 mg/kg per day of bezafibrate for two weeks and found an increase of 3.5- to 4-fold in volume density of peroxisomes in rat liver in contrast to 1.6-fold augmentation in monkeys. Studies with primary cultured hepatocytes from humans showed almost no increase of peroxisomal β-oxidation enzymes compared with up to 20-fold elevation in hepatocytes from rats after treatment with a variety of peroxisome proliferators [53]. These species differences have been attributed to differences in both quantity and quality of PPARα. In species, lacking adverse effects of peroxisome proliferators, the presence of a truncated or mutated inactive form of PPARα could be found [14], although, the presence of a full-length functional PPARα from human liver has also been described [54], albeit in much lower amount. The expression of functional PPARα in non-responders, is tenfold lower compared with high responsive species [55]. Therefore, it was suggested that PPARα expression in livers of non-responsive species may be sufficient to mediate the pharmacological effects, but not to fully activate the genes involved in peroxisome proliferation and hepatocyte growth [56]. This hypothesis could explain the observed pharmacological effects of K-111, with only slight proliferation of peroxisomes and increase in liver weight in young adult monkeys. Moreover, structural and functional differences in the peroxisome proliferator response elements (PPREs) of several genes associated with PPARα activation have been described [57,58], leading to differences in the quality of PPARα mediated responses which could play a role in explaining species specific responses to peroxisome proliferators. Interindividual differences in PPARα structure and thus in the amount of functional PPARα in non- or low responding species have been reported [14] which could account for the interindividual differences observed. The participation of essential and non-redundant cofactors and coactivators in the transcriptional activation of nuclear receptors after ligand binding has been suggested to enhance the transcriptional activity [59,60]. Indeed, a transcriptionally active PPARα interacting cofactor (PRIC) complex has been identified, which interacts with full-length PPARα after ligand binding. The PPARα–PRIC complex interacts with the response elements of the PPARα target genes. Differences in the composition of this complex may also explain the tissue and species

specific differences due to treatment with peroxisome proliferators [61].

PPARα activators are considered as key regulators of intra- and extracellular lipid and lipoprotein metabolism with potent lipid-lowering activities (reviewed in [62]). It has been suggested that reducing intracellular triglycerides and steatosis in non-adipose tissue cells improves insulin sensitivity [63]. PPARα also regulates gene transcription of key proteins involved in atherogenesis, local vascular inflammation as well as in thrombus formation (reviewed in [64]). Therefore, PPARα activators such as K-111 probably possess additional anti-atherogenic effects.

Taken together, K-111 appears to be an attractive and promising agent in the treatment of type 2 diabetes, dyslipidaemia, obesity and the metabolic syndrome, justifying clinical studies in humans.

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