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Chronic exposure to low doses of pharmaceuticals disturbs the hepatic expression of circadian genes in lean and obese mice

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ABSTRACT

Drinking water can be contaminated with pharmaceuticals. However, it is uncertain whether this contamination can be harmful for the liver, especially during obesity. Hence, the goal of our study was to determine whether chronic exposure to low doses of pharmaceuticals could have deleterious effects on livers of lean and obese mice. To this end, lean and ob/ob male mice were treated for 4 months with a mixture of 11 drugs provided in drinking water at concentrations ranging from 10 to 10^6 ng/l. At the end of the treatment, some liver and plasma abnormalities were observed in ob/ob mice treated with the cocktail containing 10^6 ng/l of each drug. For this dosage, a gene expression analysis by microarray showed altered expression of circadian genes (e.g. Bmal1, Dbp, Cry1) in lean and obese mice. RT-qPCR analyses carried out in all groups of animals confirmed that expression of 8 different circadian genes was modified in a dose-dependent manner. For some genes, a significant modification was observed for dosages as low as 10^2–10^3 ng/l. Drug mixture and obesity presented an additive effect on circadian gene expression. These data were validated in an independent study performed in female mice. Thus, our study showed that chronic exposure to trace pharmaceuticals disturbed hepatic expression of circadian genes, particularly in obese mice. Because some of the 11 drugs can be found in drinking water at such concentrations (e.g. acetaminophen, carbamazepine, ibuprofen) our data could be relevant in environmental toxicology, especially for obese individuals exposed to these contaminants.

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Introduction

Contamination of air, soil and water with pharmaceuticals and personal care products is currently a major concern for many countries. Indeed, such contamination could endanger the health of millions of individuals, especially in the case of chronic and multiple exposures in sensitive populations (Sanderson, 2011). Regarding pharmaceuticals, it is noteworthy that the parent drugs and their metabolites are excreted by patients as waste and some people also get rid of unused pharmaceuticals in the toilets. Hence, all these pharmaceuticals can thereafter be found in water (Kasprzyk-Hordern et al., 2008; Mompelat et al., 2009). For instance, the following drugs were detected in tap water in several independent studies: acetaminophen (APAP), bezafibrate and other fibrates, caffeine, carbamazepine, cotinine (a nicotine metabolite), diazepam, diclofenac, erythromycin, flumequine, ibuprofen, phenazone (antipyrine), roxithromycin, salicylic acid and sulfamethoxazole (Daughton and Ternes, 1999; Halling-Sorensen et al., 1998; Heberer, 2002; Mompelat et al., 2009; Stackelberg et al., 2004; Webb et al., 2003; Ye and Weinberg, 2007; Yu et al., 2007; Zuccato et al., 2000). As reported in most of these investigations, these drugs were found in drinking water at concentrations ranging generally between 1 and 20 ng/l. However, for some pharmaceuticals such as APAP, caffeine, carbamazepine, fibrates, ibuprofen and phenazone, several hundreds of ng/l were sometimes found in tap water (Daughton and Ternes, 1999; Mompelat et al., 2009).

Long-term exposure to drug contaminants could have deleterious consequence for some sensitive tissues, especially the liver. Indeed, liver expresses high levels of cytochromes P450 (CYPs) that can transform drugs into toxic metabolites, which are able to induce oxidative stress, mitochondrial dysfunction and cell death (Aubert et al., 2012; Leung et al., 2012). It is also worthy to mention that recent investigations indicated

Abbreviations: Alas1, aminolevulinic acid synthase 1; ALT, alanine aminotransferase; APAP, acetaminophen; Arntl, aryl hydrocarbon receptor nuclear translocator-like; Arrdc3, arrestin domain containing 3; AST, aspartate aminotransferase; Atp2b2, ATPase calcium transporting, plasma membrane 2; Bmal1, brain and muscle ARNT-like 1; Clock, circadian locomotor output cycles kaput; Cry1, cryptochrome 1; CYPs, cytochromes P450; Dbp, D site albumin promoter binding protein; GEO, gene expression omnibus; GSEA, gene set enrichment analysis; H&E, hematoxylin-eosin; Mt, metallothionein; Npas2, neuronal PAS domain protein 2; Per, period circadian clock; RT-qPCR, real-time quantitative PCR; Usp2, ubiquitin specific peptidase 2.

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that the liver could be particularly sensitive to drug-induced toxicity in the context of obesity (Aubert et al., 2012; Fromenty, 2013).

Taking all these data into consideration, the aim of the present study was to determine the long-term hepatic effects of pharmaceutical contaminants in lean and obese ob/ob mice. These mice were treated for 4 months with a mixture of 11 drugs provided in drinking water at concentrations ranging from 10 to 10^6 ng/l. These drugs included APAP, caffeine, carbamazepine, cotinine, diclofenac, erythromycin, ibuprofen, phenazone, roxithromycin, salicylic acid and sulfamethoxazole. Importantly, all these molecules were previously detected in drinking water, as mentioned previously.

Materials and methods

Animals and exposure to drugs. Five-week-old male C57BL/6J-+/+ mice (wild-type, also referred to as lean mice) weighing 19 to 20 g and C57BL/6J-ob/ob mice, weighing 28 to 32 g, were purchased from Janvier (Le-Genest-St-Isle, France) and housed in the animal house facility of Rennes 1 University under a 12 h light–dark cycle. All mice were fed ad libitum on a normal diet providing 2820 kcal per kg of food (A04 biscuits; UAR, Villemoisson-sur-Orge, France). After 1 week of acclimatization, wild-type and ob/ob mice were further split into 7 different groups that were treated or not with a drug cocktail containing 11 molecules at the following concentrations: 10, 10^2, 10^3, 10^4, 10^5 and 10^6 ng/l. All these drugs (APAP, caffeine, carbamazepine, cotinine, diclofenac, erythromycin, ibuprofen, phenazone, roxithromycin, salicylic acid and sulfamethoxazole) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Considering the molecular weight of the molecules, 10^6 ng/l corresponded to concentrations ranging from 1.2 μM for roxithromycin to 7.2 μM for salicylic acid. In a second independent series of investigations, five-week-old female C57BL/6J-+/+ and C57BL/6J-ob/ob mice were purchased from Janvier (Le-Genest-St-Isle, France) and housed in the animal house facility of the Robert Debré Hospital. In this study, mice were treated or not with the same cocktail containing the 11 drugs at the following concentrations: 10^4, 10^5 and 10^6 ng/l. For both studies conducted in Rennes and Paris, mice were exposed during 4 months to these drugs by way of the drinking water, which was renewed every week. Because ob/ob mice are drinking more than lean mice (Fromenty et al., 2009; Massart et al., 2012), drug concentrations in the drinking water were adapted in the group of obese mice to keep constant the daily intake of the drugs between lean and obese mice. Consumption of water was monitored every week and it was not reduced by the presence of the mixture, whatever the drug concentrations. On the last day of the treatment, blood was drawn in the morning between 10 a.m. and 2 p.m. Blood withdrawal was carried out from the retro orbital sinus with heparinized capillary Pasteur pipettes for biochemistry analyses. Mice were then sacrificed by cervical dislocation and the liver was quickly removed. While a majority of the liver fragments were immediately frozen in liquid nitrogen some of them were rapidly processed for appropriate histological staining. Collected tissues frozen in liquid nitrogen were subsequently stored at −80 °C until use. All experiments were performed according to the national guidelines for the use of animals in biomedical research and approved by the local Ethics Committee in Animal Experiment of Rennes 1 University and Robert Debré Hospital.

Plasma analyses. Immediately after collection, blood was centrifuged for 10 min at 1000 g and plasma was stored at −20 °C until assay. Plasma activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), total cholesterol, triglycerides and glucose levels were measured on an automatic analyzer AU2700 (Olympus Diagnostics, Rungis, France) with Olympus commercial Kits OSR6107, OSR6109, OSR6116, OSR6133 and OSR6121, respectively.

Liver histology. To evaluate necrosis, inflammation and steatosis, liver fragments were fixed in 10% neutral formalin and embedded in paraffin. Then, 4-μm thick sections were cut and stained with hematoxylin-eosin (H&E). All these sections were thoroughly examined by an experienced pathologist (V.T.-S.). The amount of hepatic steatosis in ob/ob and the classification of this lesion into 3 different categories (i.e. microvesicular, mediovesicular and macrovacuolar) were determined as recently described (Trak-Smayra et al., 2011).

RNA extraction and gene expression profiling. For the microarrays, total RNA extraction, linear T7-based amplification step, hybridization procedure, detection and read out of the fluorescence signals were carried out by Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Total RNA was extracted from liver samples using a standard RNA extraction protocol (Trizol) and quality-checked with an Agilent 2100 Bioanalyzer platform. All RNA samples revealed RNA Integrity Number (RIN) values between 7.2 and 8. Further information on T7-based amplification, Cy3-labeling, hybridization of the Agilent microarray and detection of the fluorescent signals is given in Supplementary data.

Analysis and data mining of microarray data. Gene expression data were analyzed using Feature Extraction and GeneSpring software (Agilent Technologies) and further analyzed using R-based array tools, as

Table 1
Body weight gain and plasma parameters in the different groups of mice at the end of the treatment.

Animals	Treatment	Body weight gain (g)	Glucose (mM)	Total cholesterol (mM)	Triglycerides (mM)	ALT (UI/l)	AST (UI/l)
Lean	0	11.1 ± 0.8	9.6 ± 0.9	2.49 ± 0.12	1.42 ± 0.17	86 ± 15	194 ± 50
	10 ng/l	9.8 ± 2.1	9.2 ± 0.6	2.21 ± 0.09	1.00 ± 0.09	90 ± 13	147 ± 25
	10^2 ng/l	11.4 ± 0.7	8.8 ± 0.4	2.28 ± 0.08	1.21 ± 0.16	76 ± 9	164 ± 30
	10^3 ng/l	10.5 ± 0.4	9.4 ± 0.4	2.31 ± 0.09	1.15 ± 0.08	113 ± 26	187 ± 24
	10^4 ng/l	11.9 ± 0.5	10.0 ± 0.4	2.43 ± 0.05	1.29 ± 0.10	71 ± 5	145 ± 23
	10^5 ng/l	12.8 ± 0.3	10.2 ± 0.6	2.67 ± 0.08	1.34 ± 0.03	147 ± 27	189 ± 14
	10^6 ng/l	11.3 ± 0.6	9.9 ± 0.5	2.53 ± 0.09	1.35 ± 0.18	101 ± 13	159 ± 24
ob/ob	0	30.4 ± 0.8	7.2 ± 0.7	7.88 ± 0.38	1.27 ± 0.12	708 ± 50	451 ± 24
	10 ng/l	27.7 ± 1.5	10.2 ± 3.1	7.43 ± 0.65	1.12 ± 0.09	672 ± 62	674 ± 105
	10^2 ng/l	31.7 ± 1.6	8.1 ± 0.9	7.81 ± 0.29	1.06 ± 0.02	674 ± 75	587 ± 85
	10^3 ng/l	30.0 ± 0.6	9.5 ± 1.2	7.73 ± 0.44	1.16 ± 0.08	533 ± 58[a]	372 ± 42
	10^4 ng/l	31.3 ± 0.9	8.9 ± 0.7	8.16 ± 0.32	1.19 ± 0.08	683 ± 43	748 ± 230
	10^5 ng/l	33.3 ± 0.5	9.8 ± 1.0	8.46 ± 0.29	1.07 ± 0.03	844 ± 104	759 ± 133
	10^6 ng/l	29.1 ± 0.9	9.4 ± 0.5	8.83 ± 0.28[a]	1.29 ± 0.05	936 ± 56[a]	702 ± 76
Two-way ANOVA analysis		G, T		G, T		G, T, GXT	G

Note: Plasma parameters were determined in the fed state. Body weight gain was calculated for each mouse over the 4 months of treatment. Results are mean ± SEM for 5 to 7 mice. Statistical analysis was performed with a two-way ANOVA: G, effect of genotype, T, effect of treatment, GXT, interaction between genotype and treatment. Individual means were then compared with the post hoc Bonferroni test.

[a] Significantly different from untreated mice of the same genotype ($P < 0.05$).

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