Altered xanthine oxidase and N-acetyltransferase activity in obese children

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- It has been well established that obese adults exhibit differences in expression and regulation of drug metabolizing enzymes as compared with healthy-weight adults. However, as obesity is becoming a more prevalent problem in children, little, if any, information is available that specifically addresses the consequences of obesity on drug metabolism in this population.

WHAT THIS STUDY ADDS

- This is the first study that provides evidence for elevated levels of xanthine oxidase and N-acetyltransferase 2 enzyme activity in children who are obese. This new knowledge may provide insight into improving dosing strategies in obese children with concomitant diseases who are treated with therapeutic agents that are metabolized by these enzymes.

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AIMS

It is well established that oxidative and conjugative enzyme activity differs between obese and healthy-weight adults. However, the effect of obesity on drug metabolism in children has not been studied extensively. This study examined whether obese and healthy-weight children vary with respect to oxidative enzyme activity of CYP1A2, xanthine oxidase (XO) and conjugative enzyme activity of N-acetyltransferase 2 (NAT2).

METHODS

In vivo CYP1A2, XO and NAT2 activity was assessed in obese ($n = 9$) and lean ($n = 16$) children between the ages of 6–10 years using caffeine (118.3 ml Coca Cola®) as probe. Urine samples were collected in 2-h increments over 8 h. Caffeine and metabolites were measured using LC/MS, and urinary metabolic ratios were determined based on reported methods.

RESULTS

Sixteen healthy-weight and nine obese children were evaluated. XO activity was elevated in paediatric obese volunteers compared with non-obese paediatric volunteers (XO metabolic ratio of 0.7 ± 0.06 vs. 0.6 ± 0.06 , respectively, 95% CI 0.046, 0.154, $P < 0.001$). NAT2 activity was fivefold higher in the obese (1 ± 0.4) as compared with non-obese children (0.2 ± 0.1), 95% CI 0.26, 1.34, $P < 0.05$. However, no difference was observed in CYP1A2 activity between the groups (95% CI -2.72 , 0.12 , $P > 0.05$).

CONCLUSIONS

This study provides evidence that obese children have elevated XO and NAT2 enzyme activity when compared with healthy-weight controls. Further studies are needed to determine how this may impact the efficacy of therapeutic agents that may undergo metabolism by these enzymes.

Introduction

The effect of obesity on the activity of conjugative and oxidative enzymes involved in drug metabolism has been studied in adults to some extent. Specifically obese adults exhibited a 35%, 38% and 36% decrease in clearance of CYP3A4/5 substrates carbamazepine, methylprednisolone and triazolam respectively [1–4]. However, activities of other CYP isoforms (e.g. CYP2C19, CYP2E1 and CYP2D6) were increased as compared with non-obese adults [5–8]. Studies of conjugative enzymes using lorazepam and oxazepam as probes demonstrated that obese adults also have an enhanced capacity for biotransformation via the glucuronide conjugation pathway and that these changes were proportional to total bodyweight (TBW) [9].

In contrast to the current knowledge of the effect of obesity on drug metabolism in adults, little is known about the impact of obesity on drug metabolism in children. With the prevalence of paediatric obesity approaching 30% [10–13], extensive knowledge of how each enzyme is affected is needed to begin to understand the impact of obesity on the pharmacokinetics of therapeutic agents in the paediatric population. Caffeine phenotyping in children is a successful non-invasive tool for the measurement of oxidative and conjugative drug metabolism enzymes cytochrome P450 1A2 (CYP1A2), xanthine oxidase (XO) and N-acetyltransferase 2 (NAT2; Figure 1). Specifically, this probe has been utilized in paediatric populations to determine the effects of disease on oxidative and conjugative capacities in these individuals [14–16]. The current study

evaluated the effect of obesity on drug metabolism in children using caffeine to assess CYP 1A2, NAT2 and XO activity.

Methods

The study was approved by the Institutional Review Board (IRB 0610M94328) and was conducted at the Clinical and Translational Sciences Institute of the University of Minnesota. All subjects, 7 years of age and older, signed an informed assent, where as all parents/guardians signed an informed consent which was also approved by the IRB. The participants were included based on the following criteria: (i) pre-pubertal children between ages 6–10 years; (ii) children with BMI less than 85th percentile (control) or greater than 95th percentile (obese) for age and gender (<http://www.cdc.gov/obesity/childhood/defining.html>); (iii) children not allergic to caffeine; and (iv) children currently not on any medication that interferes with drug metabolism. Obese volunteers were recruited from the University of Minnesota Weight Management Clinic, whereas lean subjects were recruited randomly.

Study volunteers were asked to refrain, for 48 h prior to administration of the caffeine, from consuming any substances that would alter basal phase I and phase II enzyme activity. Baseline saliva, blood and overnight urine samples were collected prior to the start of study. Following administration of caffeine (11.5 mg as 118.3 ml Coca Cola®) urine samples were collected in 2-h increments over 8 h [15]. Whole body dual-energy X-ray absorptiometry (DEXA)

Figure 1

Caffeine metabolism

scans were performed on each study subject to assess total body fat composition.

Quantification of caffeine metabolites

Concentrations of caffeine and its metabolites were determined using LC/MS as previously described by Weimann *et al.* [17]. Briefly, caffeine and metabolites were separated on a Phenomenex fusion reverse phase (50 mm × 3.0 mm, 3.5 µm) at 30°C. The separation was performed by gradient elution starting at 5% acetonitrile increasing to 30% acetonitrile over 4 min at a flow rate of 0.3 ml min⁻¹. Electrospray ionization was performed in the positive ion mode to detect caffeine and in the negative mode to detect the 17U (1,7-dimethyluric acid), 1X (1-methylxanthine), AFMU (5-acetylamino-6-formylamino-3-methyluracil) and 1U (1-methyluric acid) metabolites. The ([M + H]⁺/[M – H][–]) ions were selected by the first mass filter for all analytes. The coefficient of variation was less than 7.5% for all metabolites at all concentrations. The accuracy was 90–105%. Calibration curves for caffeine and metabolites were linear in the range of 2.5–2500 ng ml⁻¹. The lower limit of quantification for caffeine, 17U, 1X, 1U and AFMU metabolite was 2.5 ng ml⁻¹.

Assessment of drug metabolizing enzyme activity

Based on previously reported studies, the effect of obesity on CYP1A2, XO and NAT2 enzyme activities were evaluated using urinary metabolic ratios (MRs) of metabolites of caffeine. Specifically, CYP1A2 activity was assessed as MR (Equation 1) [18]. NAT2 activity was assessed according to MR (Equation 2) [19]. XO activity was determined using the MR (Equation 3) [20]. For all the enzymes of interest, the MR was defined as concentration of metabolite to concentration of drug. Thus, a higher MR is associated with increased enzyme activity.

$$\frac{\text{AFMU} + 1\text{U} + 1\text{X}}{17\text{U}} \quad (1)$$

$$\frac{\text{AFMU}}{\text{AFMU} + 1\text{U} + 1\text{X}} \quad (2)$$

$$\frac{1\text{U}}{(1\text{U} + 1\text{X})} \quad (3)$$

DNA isolation and genotype determination

Genomic DNA was isolated from peripheral blood lymphocytes using standard techniques [21]. DNA quantitation was carried out by measuring the absorbance at 260 nm.

NAT2 and CYP1A2 single nucleotide polymorphisms (SNPs) were determined by TaqMan from Applied Biosystems (Foster City, CA, USA). The PCR and probe primers were designed by Applied Biosystems Inc. for the following NAT2 polymorphisms: rs1208, rs1801279, rs1801280, rs1799929, rs1799930 and rs1041983 and CYP1A2 poly-

morphisms: rs762551, rs12720461, rs2069514, rs2069526, rs2470890 and rs28399424. All reactions and analyses were conducted in a 96-well plate format. The reaction components for each genotyping reaction were as follows: 1 µl of DNA (20 ng µl⁻¹), 12.5 µl of TaqMan master mix (Applied Biosystems, Branchburg, NJ, USA), 0.625 µl of primer/probe mix (Applied Biosystems, Foster City, CA, USA) and water up to a total volume of 25 µl. The thermocycler conditions were as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 92°C–15 s and 60°C–1 min. The reaction was then analysed on an Applied Biosystems PRISM model 7500 sequence detection system and software.

Obesity biomarker determination

Plasma obesity biomarkers were determined using the Human Obesity MultiAnalyte Profiling Base Kit purchased from R&D Biosystems (Minneapolis, MN, USA) following manufacturer's instructions.

Statistical analysis

Values are expressed as the mean ± SD. The difference between the means of the groups was tested for significance using Student's *t*-test. The level of statistical significance was set at *P* < 0.05. Based upon our sample size of 20 we had >90% power to detect a minimal difference 0.06 difference in XO activity, 87% power to detect a minimal difference of 0.72 for NAT2 activity and 47% power to detect a minimal difference of 1.35 for CYP 1A2 activity.

Results

Participants' characteristics

Twenty-five paediatric volunteers between ages 6–10 years were recruited for the study. Of these 25, nine were classified as obese [% total body fat (TBF) >38], while 16 were classified as lean (% TBF <25%). The median (range) age of volunteers in both groups was 9 years (6–10 years). The male:female ratio in the lean population was 7:9 while in the obese population it was 2:7 (Table 1). Laboratory tests confirmed that study subjects had no liver or kidney dysfunction. Five out of 25 study subjects were excluded from the NAT2 analysis because they were classified as rapid acetylators (NAT2*4), and thus could not be compared with the individuals who were slow acetylators (NAT2*5, NAT2*6 and NAT2*14) based upon genotype. Additionally, five study subjects were excluded from the CYP1A2 analysis because of the presence of CYP1A2 polymorphisms that conferred a reduced activity genotype. Caffeine was well tolerated by all participants and no adverse events were observed.

Biomarkers of obesity were measured to confirm subjects' metabolic obesity status (Table 1). As expected, the concentrations of leptin were 10-fold higher in the obese vs. lean population *P* < 0.001. Additionally, IL-6 and C-reactive protein were ~threefold and twofold higher

Table 1

Demographic distribution of volunteer subjects

Parameter	Lean	Obese	P value
n	16	9	
Age (years), median (range)	9 (6–10)	9 (8–10)	
Gender (M/F)	7/9	2/7	
Race/ethnicity			
Caucasian	6	1	
African-American	9	6	
Hispanic	1	1	
Native American	0	1	
BMI percentile	<84%	>95%	<0.01
Baseline caffeine (nM)	0.37	0.72	0.829
Interleukin-6 (pg ml ⁻¹)	0.55 ± 0.26	1.56 ± 1.16	0.063
Adiponectin (µg ml ⁻¹)	3.59 ± 0.65	3.14 ± 0.32	0.058
C-reactive protein (pg ml ⁻¹)	179.02 ± 62.67	269.31 ± 34.07	0.001
Leptin (µg ml ⁻¹)	6.56 ± 5.18	71.21 ± 15.28	<0.001

than in the lean population respectively. However, adiponectin concentrations were highly variable in both groups and were unaltered ($P = 0.06$).

Assessment of CYP 1A2, XO and NAT2 activity

The MR for XO in the obese group ($n = 8$) was 0.7 ± 0.06 whereas the MR for XO in the non-obese group ($n = 16$) was 0.6 ± 0.047 , 95% CI 0.046, 0.154, $P < 0.001$. This suggests that obese subjects exhibit an increase in XO enzyme activity when compared with non-obese subjects (Figure 2A). Similarly, the mean MR for NAT2 activity was observed to be fivefold higher in the obese group ($n = 6$) (1.01 ± 0.31 vs. 0.18 ± 0.13) when compared with the non-obese group ($n = 14$), thus indicating an increase in NAT2 activity in obese individuals, 95% CI 0.26, 1.34, $P < 0.008$ (Figure 2B). In contrast, for CYP1A2, the mean MR in obese children ($n = 7$) was found to be 5.4 ± 2.1 , whereas the MR in lean children ($n = 13$) was 6.7 ± 1.7 , 95% CI -2.72 , 0.12 , $P > 0.05$ (Figure 3).

Discussion

Approximately 20–30% of children in the USA are classified as obese. Obesity is a major health concern, with various epidemiological studies linking obesity to coronary heart disease, stroke, type II diabetes and several forms of cancer [22]. Limited data are available regarding the impact of obesity on pharmacokinetics of drugs in children. However, obesity has been demonstrated to affect overall outcomes associated with the treatment of paediatric neurological disorders and cancer [23–25]. Because drugs can be metabolized by different drug metabolizing enzymes, extensive knowledge of how each enzyme is affected and the drugs metabolized by that enzyme is needed to understand fully the impact of obesity on drug metabolism of a

Figure 2

Metabolic ratio (mean +SD) of XO (A) and NAT2 (B) in obese and lean subjects. XO and NAT2 activity was determined by the following caffeine and metabolite ratios [1U:(1U + 1X)] and [AFMU : (AFMU + 1X + 1U)], respectively. Study subjects included in the NAT2 activity analysis expressed slow acetylating genotype. * $P < 0.05$ comparing lean and obese at each time point. Lean (□); Obese (■)

particular agent. This is the first study to examine the effect of childhood obesity on CYP1A2, xanthine oxidase and NAT2 activity as measured by administration of caffeine as a probe drug.

XO catalyses metabolism of endogenous substrates hypoxanthine and xanthine as well as the bioconversion of the exogenous anti-neoplastic agents, such as 6-mercaptopurine. We observed that obese children exhibited a 16% increase in XO activity compared with lean controls (Figure 2A). This observed increase is relatively small compared with clinical data examining the pharmacokinetics of 6-mercaptopurine in lean and overweight paediatric cancer patients with acute lymphoblastic leukaemia. Specifically, these studies have demonstrated that obese (defined as BMI >75%) paediatric cancer patients exhibited a twofold lower overall exposure to 6-mercaptopurine as compared with the lean children (defined as BMI <75%) [26]. Even as such the magnitude of

Figure 3

Box plot of metabolic ratio for CYP1A2 activity in obese and lean subjects. CYP1A2 activity was determined by (AFMU + 1U + 1X) : 17 U. Study subjects included in CYP1A2 analysis expressed normal activity genotype

difference in 6-mercaptopurine exposure as it pertains to obesity maybe in synergy with extrinsic factors (e.g. cancer) which also have the ability to alter the activity of drug metabolism enzymes [27]. Albeit not conclusively, our finding still supports the hypothesis that increases in XO activity in obese subjects may play a role in the observed reduced exposure of 6-mercaptopurine in this population. A possible mechanism by which obesity can alter XO activity is through elevated cytokine concentrations. Cloning and characterization of the xanthine dehydrogenase gene revealed that there are cytokine-specific regulatory response elements located in the promoter region of this gene. Specifically, there were (3) IL-6 (5) INF- γ (1) NF κ -B (1) IL-1 and (1) TNF responsive elements identified [28]. *In vitro* studies in human mammary epithelial cells demonstrated that incubation with inflammatory cytokines increases xanthine oxidase activity two- to eightfold. [29] Furthermore, clinical studies quantifying the XO activity in the COPD airway epithelial lining fluid determined that XO activity was increased fourfold in COPD subjects when compared with non-COPD subjects and this elevation in XO was highly correlated with increased concentrations of the pro-inflammatory cytokines TNF- α and IL- β [30]. We observed in our population that obese children had elevated IL-6, CRP and leptin concentrations, and decreased adiponectin concentrations when compared with the lean controls (Table 1). Therefore, it is possible that these pro-inflammatory cytokines and adipokines which are excessively produced in the state of obesity upregulate XO gene expression and activity in these individuals. Another explanation for the increased XO activity maybe because of an increase liver volume associated with obesity, thereby resulting in a higher metabolic activity in obese children when compared with lean children. Even though plausible, studies evaluating the pharmacokinetics and disposition of the CYP3A4/5 substrates carbam-

azepine, methylprednisolone and triazalam reported that obese subjects exhibited a decrease in clearance of these substrates by 35%, 38%, 36%, respectively, when compared with non-obese adults [1, 3, 4].

NAT2 is responsible for the metabolism of some xenobiotics (e.g. metabolism isoniazid and procainamide) and the activation of some carcinogens (e.g. aromatic and heterocyclic amines). Additionally, this enzyme is responsible for the formation of the caffeine metabolite 5-acetylamino-6-formylamino-3-methyluracil (AFMU). Among the slow acetylators, we observed a fivefold increase in NAT2 activity in the obese as compared with non-obese children (Figure 2B). NAT2 activity has been reported to be associated with elevated risk of colon rectal cancer and breast cancer [31–33] in individuals who have slow acetylator phenotype. Additionally, there is evidence that suggests that obese adults and children have increased cancer risks compared with non-obese counterparts [34–36]. This increase in cancer risk may be associated with alterations in oxidative and conjugative enzyme capacity for activating or inactivating carcinogenic agents. Thus, the contribution of obesity to elevated enzyme activity may also be a predictor of increased risk for overall exposure to carcinogens activated through the NAT2 pathway.

CYP1A2 makes up approximately 10% of total liver CYP content [37] and is responsible for the metabolism of approximately 4% of drugs used [38]. Common drugs that are substrates for the CYP1A2 isozyme include R-warfarin, theophylline, caffeine, some benzodiazepines, antidepressants and antipsychotics. We observed that there was no difference in CYP1A2 enzyme activity between obese and non-obese children (Figure 3). Murine studies in the genetically obese mice (ob/ob) observed that obese mice exhibit reduced CYP1A activity when compared with lean controls [39]. However, evidence of an effect of obesity on CYP1A2 activity is inconclusive [40]. Alterations in both NAT2 and XO activity and not CYP1A2 activity demonstrates that the increase in metabolic ratio in the obese vs. non-obese is independent of the conversion of caffeine to the paraxanthine metabolite. Bracco *et al.* determined that parent caffeine urinary excretion was not different in lean vs. obese women. However, urinary caffeine metabolites were more abundant in the obese women when compared with lean women. This suggests that dissimilarities in the production and excretion of these caffeine metabolites in obese vs. lean women may be a result of altered expression or activity of enzymes involved in caffeine metabolism similar to what was observed in our paediatric population [41].

Despite our findings, our study does present some limitations, the first limitation being with respect to the MRs used to determine CYP1A2, NAT2 and XO activities. The MRs to determine CYP1A2, NAT2 and XO activities depend primarily on the urine collection time. Studies have shown that it is imperative to collect urine for long enough time periods so that caffeine metabolites can be formed. Rost

et al. determined that the 5–8 h (AMFU + 1U + 1X:17U) urinary ratio is a better correlative measure of CYP1A2 than using urine collection times greater than 24 h because it is independent of urine flow rate [42]. Furthermore, Kennedy *et al.* determined that 8-h urine collection time is sufficient for paediatric phenotyping [15, 42]. Another limitation of the study is the inability to ascertain additional environmental factors (e.g. smoking, diet, exercise) that have been shown to be associated with altered enzyme activities [43, 44].

In conclusion, XO and NAT2 enzyme activities are elevated in obese children. Future studies should be pursued to determine other drug metabolism enzymes that may be altered by obesity in children. Additionally, studies should also be undertaken to determine the clinical implications of altered drug metabolism. This will enable us to develop dosing models that will help optimize dosing regimens in obese children with concomitant diseases.

Competing Interests

There are no competing interests to declare.

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