Short Communication

THE EFFECT OF AGE ON SILDENAFIL BIOTRANSFORMATION IN RAT AND MOUSE LIVER MICROSONES

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ABSTRACT:

Sildenafil [SIL (Viagra); Pfizer, New York, NY] is a widely prescribed agent for erectile dysfunction in men older than 65 years. The present study evaluated experimental models to assess age-dependent changes in SIL biotransformation using hepatic microsomes from male rats and mice ranging from 6 weeks to 26 months of age. The role of specific isoforms in the conversion of SIL to its primary circulating metabolite, UK-103,320 (piperazine N-desmethyl sildenafil) in the mouse was also investigated using immunoinhibitory antibodies. Although CYP2C11 largely mediated UK-103,320 formation in the rat, UK-103,320 formation was principally inhibited by a CYP3A antibody in the mouse. An age-related decrement in metabolite formation rate was observed for both species, although this effect was more pronounced in the old rats (reduced to 7% of young) than in the old mice (reduced to 51% of young). CYP2C expression was assessed by Western blot analysis in rat and mouse livers. Age-related differences in hepatic CYP3A expression in the mouse were also compared with metabolite formation rates in the mouse model. Decrements with age in CYP2C and -3A expression in the aging rodents paralleled the decrements in SIL biotransformation, suggesting that age-related differences in SIL metabolic rate may, in part, reflect differences in expression. Although the role of specific CYP enzymes and the clearance values for this reaction may differ among species, age-related changes in these rodent models are consistent with the reduced clearance of SIL observed in human studies.

Erectile dysfunction (ED) is prevalent in the elderly (Feldman et al., 1994; Rhoden et al., 2002). Sildenafil [SIL (Viagra); Pfizer, New York, NY], a cGMP phosphodiesterase type 5 inhibitor, is a widely prescribed treatment for male ED and may be efficacious in this population (Wagner et al., 2001). Given the prevalence of ED in the elderly population and the widespread use of SIL for ED treatment, the study of age-related differences in the pharmacokinetics of SIL is critical.

Muirhead et al. (2002) demonstrated a 60 to 70% increase in maximum SIL concentrations in older male subjects, as well as oral clearance values approximately half those observed in young male subjects. The factors that contribute to these age-related changes in pharmacokinetic parameters are not clearly established.

In humans, the conversion of SIL to its principal circulating metabolite, UK-103,320, is largely mediated by cytochrome P450 3A (CYP3A) (Warrington et al., 2000a; Hyland et al., 2001), the most abundantly expressed member of the cytochrome P450 superfamily in the liver (Maurel, 1996). Although the age-related decrement in SIL clearance may be attributable to an age-related decline in the metabolic activity or expression of CYP3A in humans, in vitro studies on the effect of age on CYP3A activity and expression in humans have been largely inconclusive (Warrington et al., 2000b). Differences among studies may be attributable, in part, to study design and analysis, such as differences in tissue procurement and storage, as well as to other potentially confounding factors such as concomitant medications, comorbid diseases, diet, smoking, and genetic variability (Schmucker et al., 1990; Transon et al., 1996).

A few studies have used animal models to control for some of the variability observed in aging in vitro human studies (Barnhill et al., 1990; Warrington et al., 2000b). However, to our knowledge, the effect of age on SIL biotransformation in rodent models has not been examined.

Formation of the major SIL metabolite, UK-103,320, was observed in the mouse, dog, rat, and human (Muirhead et al., 1996; Walker et al., 1999). Plasma clearance of SIL was significantly higher in the mouse and male rat than in the dog, female rat, and humans (Walker et al., 1999). These species differences may be attributable, in part, to differences in metabolic pathways (Warrington et al., 2002). For example, whereas SIL biotransformation is largely CYP3A-mediated in humans, it is mediated by CYP2C11 in the male rat. To our knowledge, the contribution of specific cytochromes P450 to murine SIL biotransformation has not been established. Therefore, we examined the metabolic pathways that contribute to UK-130,320 formation in the mouse and evaluated the effect of age on SIL biotransformation in both the rat and mouse.

Materials and Methods

Thirty-two male Fischer 344 rats were obtained from the National Institute of Aging (held at Harlan, Indianapolis, IN) (N. Nadon, Office of Biological Resources and Resource Development, National Institute on Aging, personal communication). Animals were housed at the Tufts University Animal Facilities for 1 to 2 months before sacrifice. Twenty-eight healthy rats (young: 3–4 months, n = 10; intermediate: 13–14 months, n = 10; old: 25–26 months, n =...
A, increasing concentrations of SIL (0–63 μM) were incubated with liver microsomes (0.05 mg/ml) from young (n = 10), intermediate (n = 10), and old (n = 8) rats. Lines represent functions consistent with a single-enzyme Michaelis-Menten model. B, SIL (36 μM) was incubated with young (circles; n = 6), intermediate (squares; n = 6), and old (triangles; n = 6) mouse liver microsomes. Solid lines denote the mean values. Metabolite formation rate is expressed in relative units. For both species, differences were detected among the three age groups at 36 μM (ANOVA for mouse; Kruskal-Wallis ANOVA for rat, p < 0.001). * and †, a difference of the old group from the young and intermediate (Dunn’s, p < 0.05) and a difference of the intermediate from the young and old groups (Student-Newman-Keuls, p < 0.05).

**SIL Biotransformation Using Mouse and Rat Hepatic Microsomes.** SIL (0–63 μM) was incubated with hepatic microsomes (0.05 mg/ml) in the presence of a NADPH-regenerating system for 20 min at 37°C, as described previously (Warrington et al., 2000a, 2002). For studies of rat microsomes, incubations were performed with varying concentrations of SIL (0–63 μM). For mouse microsomal studies, a single SIL concentration (36 μM) was used. Incubations were terminated by exposure to ice and acetonitrile (2:5 vol), and buspirone (4 μM) was added as an internal standard. After centrifugation (10 min), supernatants were transferred to HPLC autosampling vials. Incubation time and protein concentrations were within a linear range. Metabolite identification was based on chromatographic retention times, as described previously (Warrington et al., 2000a, 2002). Samples without protein, substrate, or cofactor did not demonstrate metabolite formation.

**Immunoinhibition of UK-103,320 Formation in Rat and Mouse Hepatic Microsomes.** SIL (36 μM) and liver microsomes (10 μg) were incubated with either 1 of 5 immunoinhibitory antibodies (anti-rat CYP3A2, -2E1, -1A1/2, -2B1/2, and -2C11; 100 μg), a serum control (goat or rabbit; 100 μg), or 50 mM potassium phosphate buffer for 30 min at 37°C. An NADPH-containing cofactor was added to initiate metabolite production. After 20 min at 37°C, reactions were terminated with acetonitrile and subjected to the same conditions described above.

**Western Blot Analysis of Rat and Murine CYP2C Isoforms.** Western blot analysis was conducted as described previously (Perloff et al., 2000; Warrington et al., 2000b). Briefly, either rat (0.38–37 μg) or mouse (0.5–1.5 μg) microsomal protein was separated on 7.5% Tris-HCl polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Membranes were then incubated with 3 to 5% milk in 1× Tris-buffered saline 0.06% Tween 20 for 30 min and probed with either an anti-rat CYP2C11 (1:500) in rat or both an anti-rat CYP2C11 (1:500) and -2C13 (1:500 or 1:4000) antibody in mouse (BD Gentest, Woburn, MA) for 1 to 2 h. After washing, membranes were then incubated with 3 to 5% milk for 45 to 60 min and probed with a donkey anti-goat IgG-horseradish peroxidase antibody (1:5000 or 1:10,000; Santa Cruz Biochemicals, Santa Cruz, CA). Membranes were washed, exposed to a chemiluminescent substrate (Pierce Chemical, Rockford, IL), and imaged.

Samples were quantified relative to serially diluted young rat or mouse hepatic microsomal standards, which were used to construct a calibration curve of net intensity (pixels × area of each band background at the band’s perim-

**Data Analysis.** UK-103,320 formation was consistent with Michaelis-Menten kinetics and was fitted to a single-enzyme model:

$$V = \frac{V_{\text{max}} S}{K_m + S}$$

in which V represents the reaction velocity, S denotes the substrate concentration, $V_{\text{max}}$ is the maximum reaction velocity, and $K_m$ is the substrate concentration at which 50% of $V_{\text{max}}$ is achieved (Venkatakrishnan et al., 2001). Since reference standards were unavailable, reaction velocities are expressed in relative arbitrary units, as previously described (Warrington et al., 2000a, 2002).

Age-related differences were evaluated using either a one-way ANOVA or a one-way Kruskal-Wallis ANOVA on ranks with Student-Newman-Keuls (parametric) and Dunn’s multiple comparisons (nonparametric) tests to evaluate differences between individual age groups. Student’s t tests were used for immunoinhibition assays.

**Results.** Using a panel of five immunoinhibitory antibodies (anti-rat CYP3A2, -2E1, -1A1/2, -2B1/2, -2C11), only the CYP3A2 antibody inhibited SIL biotransformation in mouse liver microsomes (Student’s t test, p < 0.05). This antibody reduced UK-103,320 formation to 24% of the control value.

Age-related differences in UK-103,320 formation were apparent in both the rat and mouse (Fig. 1). In rats, $V_{\text{max}}$ and intrinsic clearance values were significantly lower in old animals compared with the young and intermediate groups. Mean $V_{\text{max}}$ values were 1.7, 1.3, and 0.1 units in young, intermediate, and old rats, respectively ($F = 20.58$, $p < 0.001$). Mean intrinsic clearance values were: 247, 329, and 45 units in the three groups, respectively ($F = 12.8$, $p < 0.05$). $K_m$ values, on the other hand, did not change with age (mean values, 4–5 μM). Adjustments for microsomal protein per milligram of liver or for total liver weight did not alter the statistical conclusions. In mice, reaction velocities at 36 μM SIL also differed significantly among age groups,
with old mice having significantly lower values than the intermediate group (Fig. 1).

Previous immunoinhibition studies in young mice and rats (Warrington et al., 2002) were extended to include animals from the intermediate and old age groups. Regardless of age group, UK-103,320 formation was inhibited only by the anti-CYP2C antibody in the rat and only by the anti-CYP3A antibody in the mouse.

Using the anti-rat CYP2C11 antibody, Western blot analyses (Fig. 2) revealed two bands (∼55 kDa), that, consistent with data provided by the antibody’s manufacturers, are likely to represent CYP2C11 (the upper band) and -2C13 (the lower band), in the male rat liver. Using either the anti-rat CYP2C11 or -2C13 antibodies, only one CYP2C isoform (∼50 kDa) was identified in the mouse liver. Age-related differences were detected in CYP2C expression in both rat and mouse livers, largely reflecting a decline in CYP2C expression in the old livers. In the rat, the old livers expressed 45% of CYP2C13 and 6.7% of CYP2C11 compared with that observed in young livers. Although CYP2C expression declined with increasing age in the male rat, there was an increase in CYP2C expression in the intermediate group for the mouse. Similar age-related trends were observed for SIL biotransformation as well (Fig. 1). Differences among groups in liver weight or microsomal protein did not alter the age-related trends.

One outlier was observed among the old rats. This outlying value did not affect the age-related trends or statistical conclusions, but inclusion of this value altered the estimated magnitude of difference among the age groups for both SIL biotransformation and CYP2C expression. In both measurements, this old liver group expressed considerably higher levels (36-fold) of CYP2C11 than other old rats. When this outlier was removed from the old group, the average CYP2C11 expression for the old group fell from 0.22 ± 0.08 to 0.20 ± 0.04 (arbitrary units), which represents 1.2% of the average CYP2C11 in the young.

Net CYP2C expression and the intrinsic clearance of SIL were significantly correlated in the male rat ($r^2 = 0.78, p < 0.001$). When the expression of CYP2C11 and -2C13 were compared separately with SIL biotransformation at relative $V_{max}$ values, the high degree of correlation was maintained with CYP2C11 but was not present with CYP2C13. These findings are consistent with previous studies in which SIL biotransformation was found to be largely mediated by CYP2C11.

When SIL biotransformation in the mouse was compared with CYP3A expression values (Warrington et al., 2000b), a lower but statistically significant correlation value was identified between these two parameters ($r^2 = 0.46, p < 0.001$).

**Discussion**

This study demonstrates that although CYP2C11 may contribute to UK-103,320 formation in the male rat (Warrington et al., 2002), CYP3A isoforms may mediate murine SIL biotransformation. The role of CYP3A isoforms in murine SIL biotransformation was supported by immunoinhibition studies and moderate correlation with immunoreactive CYP3A expression. However, since the cross-immunoreactivity of the anti-rat antibodies in mouse remains unclear, further study of the role of CYP3A in murine SIL biotransformation is warranted. Nonetheless, since SIL is largely a CYP3A substrate in
humans (Warrington et al., 2000a; Hyland et al., 2001), the specific cytochrome P450 subfamily that mediates SILD biotransformation in the male mouse is likely to correspond to that in humans and is unlike that previously observed for the male rat.

In addition, this study demonstrated an age-related decline in SILD metabolism in both rodent models, although this decline was more marked in the male rat. Age-related differences in cytochrome P450 activity correlated with changes in rat CYP2C expression described in this study and in murine CYP3A expression described previously for this cohort of mice (Warrington et al., 2000b).

These findings are consistent with previous studies on age-related changes in rat CYP2C expression (Kamataki et al., 1985; Imaoka et al., 1991; Agrawal and Shapiro, 2003) and activity (Imaoka et al., 1991). However, using a male rat CYP2C11 index reaction (Warrington et al., 2002), this study affords increasingly greater specificity for the effect of age on CYP2C11 activity. Since CYP2C11 represents the most highly expressed cytochrome P450 in the male rat liver (Guengerich et al., 1982; Imaoka et al., 1990), a specific marker for CYP2C11 function is of value for aging studies.

Consistent with previous studies using other substrates (Ring et al., 1994; Eagling et al., 1998; Perloff et al., 2000), important species differences are likely to exist in vitro clearance values and in the role of specific cytochrome P450 isoforms in UK-103,320 formation. Nonetheless, age-related differences in this study are consistent with previous studies on age-related declines in drug clearance for many cytochrome P450-mediated reactions in rodents in rat (Barnhill et al., 1990) and in humans in vivo (Greenblatt et al., 1991a, b; von Moltke and Greenblatt, 1999; Cotreau et al., 2003).

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References


