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FORUM

Effects of PPARγ and Combined Agonists on the Urinary Tract of Rats and Other Species

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INTRODUCTION

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors involved in regulating lipids and the effects of insulin (Yki-Järvinen, 2004). Three major receptors have been identified, alpha, gamma and delta (beta), with differing tissue distributions and effects. Agonists have been developed for each of these receptors, with differing pharmacologic and toxicologic effects.

PPARα are present in liver, and also present in several other tissues including skeletal muscle, heart, and brown fat; their agonists have effects on maintenance of lipid levels, especially triglycerides (Klaunig *et al.*, 2003). The toxicological effects of PPARα agonists (peroxisome proliferators) have been extensively investigated, including carcinogenic effects in rodents. They induce liver tumors in rats and mice and frequently induce rat pancreatic acinar cell and testicular Leydig cell tumors. The modes of action for these tumors have been identified and generally been found not to be relevant to humans (at least quantitatively) (Klaunig *et al*., 2003).

PPARδ(β) are ubiquitous, whereas PPARγ are present in adipose tissue, endothelial cells, and several epithelial tissues, such as urothelium and intestine (Yki-Järvinen, 2004). Agonists to these receptors, either alone or combination effects on more than one PPAR receptor, are being developed for clinical use in treating lipid disorders and diabetes. The effects of PPARδ(β) agonists on carcinogenesis have not been extensively investigated, but have already produced varying and conflicting results. PPARγ agonists and dual PPARγ/α agonists have been more extensively investigated. Although various tumors have been produced by specific agonists, the most common types of tumors observed so far have been rat urinary bladder urothelial tumors and various mesenchymal sarcomas (especially in subcutaneous adipose tissue) in rats, mice, and hamsters (El-Hage, 2005). This article addresses the issue of the urothelial tumors.

Urothelial tumors in rats, particularly in the urinary bladder, have become an increasing concern with respect to predicting possible human cancer risk for PPARγ and combined agonists (El-Hage, 2005). Five of six dual PPARγ/PPARα agonists and pioglitazone were listed as having carcinogenic activity toward the rat bladder by El-Hage (2005). Another, rosiglitazone, produced rat bladder tumors in a two-stage model at a higher dose than had been used in the 2-year bioassay (Lubet *et al.*, 2004). The bladder tumors have occurred predominantly in male rats compared to females and have not been reported in mice. The difficulty in providing an assessment of these is the lack of information in the public domain. Nevertheless, information regarding the biology of these agonists with respect to the urothelium and our understanding of chemical carcinogenesis of the bladder in rats can be utilized to provide hypotheses which can be tested experimentally and evaluated with respect to potential risk for humans. PPARα agonists have not produced bladder tumors or preneoplastic urothelial changes in rats or mice in 2-year bioassays or in shorter-term studies (Klaunig *et al.*, 2003). Thus, the focus appears to be on the effects of these agonists on the PPARγ receptor.

Any mechanism addressing the bladder tumors has to explain the sex and species differences, and also to explain what appears to be a pharmacological class effect rather than merely an effect of a specific chemical or specific chemical class such as the thiazolidinediones (''glitazones''), since some of the ''glitazars'' have also had these effects.

Two hypotheses have been formulated regarding urothelial carcinogenesis by these agents, with some variations. The first of these involves a direct effect of the agents on the urothelial PPARγ receptors; the second hypothesis suggests an indirect effect of these agents, not specifically targeting the PPARγ receptor in the urothelium. There is no evidence of genotoxicity for these agents.

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DIRECT EFFECT ON PPARγ IN THE UROTHELIUM

PPARγ are nuclear receptors present in adipose tissue and also in a variety of other cell types, including the urothelium (Varley *et al.*, 2003, 2004). The extent of expression and interaction appears to vary between species, but there does not appear to be much variation between sexes within a given species (or strain). However, such conclusions are based upon a variety of methodologies, and comparisons between species and sexes are method dependent.

Since these receptors are present in the urothelium, it is natural to hypothesize that the urothelial effects of the PPARγ agonists are produced by direct interaction with the receptor in the urothelium. However, there are several observations for which this hypothesis raises considerable difficulty:

1. A direct effect on the PPARγ receptor in the urothelium does not explain the striking sex or species differences that have been observed.
2. These agonists and their pharmacologically active metabolites are extremely lipophilic and are excreted in the urine to only a limited extent.
3. Urothelial carcinogenesis is considered to be mediated through urinary exposure rather than hematogenous exposure (Clayson and Cooper, 1970).
4. Early changes and most of the tumors arise in the ventral dome of the bladder, where the urine settles in rodent species.
5. Most importantly, the biology of this receptor in the urothelium is exactly the opposite of what is anticipated for a urothelial carcinogen (Guan *et al.*, 1997; Kawakami *et al.*, 2002; Varley *et al.*, 2003, 2004). Rather than increasing cell proliferation, the PPARγ receptor is associated with potentiation of differentiation and inhibition of proliferation. An effect on apoptosis has been suggested from *in vitro* studies (Varley *et al.*, 2003, 2004), but the normal urothelium has rare apoptotic bodies (Shirai *et al.*, 1995). The proliferation of urothelial carcinoma cell lines is also inhibited (Guan *et al.*, 1999). Based on findings in the urothelium and other tissues, this class of drugs has been suggested as potential chemopreventive or anti-cancer therapeutic agents. A chemopreventive clinical trial for recurrence of bladder tumors is underway using rosiglitazone (see www.cancer.gov/clinicaltrials/fccc-03018).
6. The basic biology of the PPARγ receptor involves the formation of a heterodimer with the RXR receptor leading to its action on DNA and transcription. RXR is activated by a variety of retinoids. In both rats and mice, several retinoids showed significant anticarcinogenic and chemopreventive effects on urothelial tumors (Moon *et al.*, 1982; Sporn and Newton, 1981). Clinical trials were begun for the chemoprevention of bladder tumor recurrences, but had to be halted because of the extreme toxicity that these agents had when administered to older (age > 50) individuals (G.H. Friedell, personal communication), in contrast to the tolerance of these agents at similar doses used for the treatment of acne and other skin disorders in younger people.

In summary, although PPARγ receptors are present in the urothelium, there are a number of reasons to suggest that interaction with the urothelial receptor is not the mechanism by which these agents produce bladder tumors.

INDIRECT MODE OF ACTION INVOLVING URINARY SOLIDS

An alternative is hypothesized involving an indirect mode of action based on the pharmacologic action of these drugs in rodents and in other species. This involves altering urinary composition leading to the formation of urinary solids, a well-known mode of action, particularly in rodents, for the induction of bladder tumors, including by certain classes of drugs such as carbonic anhydrase inhibitors, HIV protease inhibitors, and sulfonamides (Clayson *et al.*, 1995; Cohen, 1998; IARC, 1999).

A characteristic effect of PPARγ and dual agonists is the induction of increased cardiac size and accumulation of body water. The FDA has accepted an increase in heart size as one determinant for a maximum tolerated dose (MTD) for this class of drugs (El-Hage, 2005). This effect is dose dependent and is a pharmacologically based response to these agents. Physiologically this and other effects will modify renal function, altering fluid dynamics, and lead to significant changes in urine composition. These composition changes ultimately lead to formation of various types of urinary solids (precipitate, microcrystals, calculi), which are known to be irritative and toxic to the urothelium, especially in rats, leading to sustained regenerative proliferation and ultimately to the induction of bladder (urothelial) tumors (Cohen, 1998, 1999; IARC, 1999). Like most urothelial carcinogens, the effect is most commonly seen in the urinary bladder, but changes can also be seen in the kidney pelvis and ureters. There is an extensive literature on the toxic and carcinogenic effects of urinary solids (Clayson *et al.*, 1995; Cohen, 1998, 1999; IARC, 1999; Rodent Bladder Carcinogenesis Working Group, 1995). In brief, it is a high-dose phenomenon for which humans are significantly less susceptible than rodents.

There are several aspects of this hypothesis that are consistent with the findings that have been reported so far for these agents:

1. Pharmacological effects on body fluids and urinary composition are a consistent finding with these agents.
2. Early changes with these agonists in the rat are observed in the ventral dome of the bladder, where urine settles.
3. In general, the production of urinary solids and associated proliferative and carcinogenic effects have been

seen more readily in male rats compared to female rats, and in rats more readily than in mice.

There are several aspects of this hypothesis that can be critically evaluated. Most important is to document that urinary solids are actually formed and to determine the composition of the solids. The urinary solids are not likely to be composed of the administered agonists and/or metabolites, since extremely small amounts are excreted in the urine. Rather, the urinary solids are expected to be composed of normally present constituents of urine (Cohen, 1998; Cohen *et al.*, 2000). Since this is an indirect effect on urine composition, the most likely urinary solids to be formed would be calcium-containing. If so, there are several aspects that have been well delineated for this process in rodents.

Urinary pH is of critical importance for the formation of calcium-containing solids (Clayson *et al.*, 1995; Cohen, 1995). This does not necessarily mean that the agents increase the urinary pH; the critical parameter is keeping the urinary pH above a certain level. This has been ascertained at approximately 6.5 in male rats, but this could vary depending upon specific agent, sex, species, and the specific type of urinary solid being formed (Cohen, 1999; Cohen *et al.*, 1995, 2000; Okamura *et al.*, 1991). Other critical factors influencing formation of calcium-containing urinary solids are concentrations of calcium, phosphate, oxalate, magnesium, citrate (a chelator of calcium), and protein (qualitative and quantitative) and osmolality.

If calcium-containing urinary solids are indeed involved, their formation can be readily prevented by administering an agent (usually ammonium chloride in the diet) that produces acidified urine (Clayson *et al.*, 1995; Cohen, 1995, 1999). Also, administering an agent that leads to a marked increase in urinary volume, such as sodium chloride, can provide an inhibitory effect by diluting the concentration of the constituents in the urine that lead to the formation of urinary solids (Okumura *et al.*, 1992). However, this is not as reliable a method of inhibiting the formation of urinary solids as is acidification.

Male rats are particularly prone to the development of the formation of these urinary solids, due in part to extremely high density (osmolality) of the urine (Cohen, 1995) and the high concentrations of urinary protein (predominantly due to α_{2u}-globulin at younger ages and albumin at older ages) (Hard, 1995). Rats are more likely to form calcium-containing urinary solids than mice, because rats have 10–20 times higher urinary concentrations of calcium, and phosphate and magnesium concentrations are also higher in rats (Cohen, 1995).

Since PPARγ and combined agonists increase fluid retention in rats, they frequently are associated with increased urine volume. This also involves increased water consumption. Based on studies with other classes of substances, such as sodium salts, increasing urine volume in rats usually is accompanied by enlargement of the bladder and frequently with urothelial cell hypertrophy. These changes in the bladder are accompanied by other metabolic and biochemical changes, which likely would be reflected in changes detectable by transcriptomics or proteomics. Such changes should not be mistakenly attributed to responses of direct interaction of the agonists with the PPARγ receptor in the urothelium.

Another significant observation for some PPARγ agonists is that early in their administration to rats there is little to no effect on the urothelium detected by light microscopy. Changes are usually seen after 6 months or up to 1 year, depending upon the agent. However, changes at earlier times are likely to be detectable using the more sensitive techniques of scanning electron microscopy (SEM) and bromodeoxyuridine (BrdU) labeling index (Cohen *et al.*, 1990). Aging nephropathy (Goldstein *et al.*, 1988) will also modify urine composition. Combined with the alterations produced by these PPARγ agonists, it will likely potentiate the effects on the urine with age and be a particularly significant effect in male rats compared to females.

In summary, a hypothesis for an indirect mode of action is consistent with what is known with these agonists: It is pharmacologically based, it explains the sex and species differences, it explains the increase in findings with respect to age, and it fits with the known effects of these agents on the receptors in the urothelium. Most importantly, it is a readily testable hypothesis.

ASSESSMENT OF URINE FOR CHEMICAL CONSTITUENTS AND URINARY SOLIDS

The assessment of the urine for compositional alterations and for detection of solids becomes a critical issue in evaluating the hypothesis. There is an extensive published experience regarding the issues that are involved (Cohen, 1995).

Unfortunately, routine examination of the urine as performed by most investigators and routinely by pharmaceutical companies will generally not result in detection of either the chemical compositional changes that are hypothesized, nor will it be useful in detecting the formation of urinary solids.

Urine is a dynamic body fluid which is changing in composition on a regular basis. The composition can be greatly influenced by exogenous conditions, especially food and water intake (Cohen, 1995). Humidity and temperature also can play a role. These points sound simple and, yet, are generally not taken into account in routine urinalysis in experimental animal studies.

Rodents are nocturnal animals, eating and drinking at night, although this routine is altered by diet-restriction protocols. Marked diurnal variations in the composition of the urine occur, reflecting ingestion of the food and water (Fisher *et al.*, 1989). All of the substances in urine change on a diurnal basis, and this affects the responsiveness of the urothelium, including diurnal variation of mitotic activity (Tiltman and Friedell, 1972).

The major implication of this diurnal variation is that collection of urine during the day, when the lights are on, is usually the worst time to be collecting urine to see treatment effects on urine composition. The timing of the urine collection is critical, taking into account not only the dark/light schedule of the laboratory, but the timing of food and water consumption, particularly if these are on a restricted basis, the timing of drug administration, particularly if by gavage as done for the PPARγ and dual agonists, the type of handling (Cohen *et al.*, 1996), and the pharmacokinetics of the drug, particularly as it involves urine concentrations. In addition, long-term collection of the urine (overnight or for 24 h) frequently can modify the composition of the urine and mask the critical changes that occur (Cohen, 1995). Long-term urine collection necessitates collection on ice and/or with preservatives, which also can artifactually influence formation of urinary solids. Fresh void urine is strongly recommended for examination. This can be readily accomplished with some practice such that 100 μl or more can be routinely collected from an early morning specimen in rats, and the animal does not have to be placed in a separate metabolism cage. Mice are obviously more difficult. Determination of the optimum time of day for collection of the urine to detect critical changes can be complicated and difficult, but is essential.

A common practice, with severe consequences on urine composition, is fasting of the animals for up to 24 h prior to, and/or food and water deprivation during the urine collection. Fasting of animals almost immediately produces marked changes in urinary composition, and by 24 h any changes that are of importance for evaluation of any hypothesis have disappeared, even the presence of calculi or other urinary solids (Cohen, 1995; Shirai *et al.*, 1995). Thus, statements concerning the absence of urinary solids in rodents treated with these agonists must be evaluated with caution until the details of urine collection and processing are assessed.

How urine is processed is also critical. Urinary pH can be assessed immediately upon collection with a single drop of urine using a microelectrode (Cohen, 1995). Dipsticks are to be avoided for pH or any other determination of rodent urine. Processing for observation of urinary solids needs to be done within an hour or two of urine collection, since many of these substances are in a steady state, and the urinary solids can dissolve in an aqueous solution such as urine (Shirai *et al.*, 1995). Examination of the bladder at the time of autopsy for urinary solids is also critical, since allowing the bladder to sit in fixative for a period of time before examination can lead to the disappearance of solids that might have been present.

Evaluation of the urothelium also can be fraught with difficulties. Routinely, the urinary bladder is removed toward the end of the necropsy and immersed in buffered formalin fixative. This is useful for detection of significant changes, but is inadequate for detection of early, relatively mild changes, such as superficial cell necrosis or increase in cell proliferation without concomitant hyperplasia. Inflation of the bladder with fixative at the time of necropsy is critical to assess minimal changes, rather than evaluating an uninflated bladder. For more subtle changes, light microscopy, BrdU labeling index (1-h pulse following ip injection), and evaluation by SEM provide a combination of techniques that detects subtle changes in the bladder, including with PPARγ and dual agonists, several weeks or months prior to detection of changes using only light microscopy (Cohen *et al.*, 1990, 1995).

Fixation is also an important issue, particularly if SEM is to be performed. Autolysis occurs rapidly in the urothelium, with morphologically detectable changes within 60 s of death (Takayama *et al.*, 1998). *In situ* inflation of the bladder with fixative while the animal is under deep anesthesia is recommended rather than waiting for the animal to die. If it is required that the animal be dead before the bladder can be taken, the bladder has to be removed first and immediately inflated with fixative.

A useful fixative is Bouin's solution; it provides adequate fixation for SEM examination and reasonable fixation for light microscopic and BrdU labeling immunohistochemical examination. Placement of a dab of India ink on the ventral surface of the bladder while it is *in situ* helps locate the ventral dome of the bladder in the evaluation by SEM, BrdU labeling index, or light microscopy.

EFFECTS ON MONKEY UROTHELIUM

There has been some information presented by FDA indicating that there are urothelial changes in the monkey (and possibly the dog) in addition to the rat (El-Hage, 2005). These include the presence of granules, vacuoles, and hyperplasia. Although these effects have not been fully evaluated, they do not appear to represent preneoplastic changes.

The granules have been documented in the literature as keratohyalin granules and are normally present in the monkey bladder, unlike the bladder in most mammalian species including humans (Burek *et al.*, 1972).

It is unclear exactly what the vacuoles are, but they may represent an artifact of autolysis that occurs in the processing of these bladders. Such vacuoles are present usually between the superficial and intermediate cell layers, and frequently contain cells with pyknotic nuclei. Like the rodent, the monkey urothelium undergoes autolysis rapidly, with morphologic changes evident by SEM within 60 s of death (Takayama *et al.*, 1998). Vacuoles have been reported in normal monkey urothelium from the renal pelvis to the urethra (Takayama *et al.*, 1999), and they appear to be present in all monkeys if routinely processed (unpublished observations). In the routine monkey autopsy, the urinary bladder is one of the last tissues to be placed in fixative, usually 15–30 min or more after the animal has died. The cells within these vacuoles are lymphocytes and macrophages, which are also cells that are normally present in the monkey urothelium but not in human or rodent urothelium.

The most difficult issue with these vacuoles is that they appear to increase in number and size with higher doses of the PPARγ or combined agonists being administered. This could be related to increasing levels of edema present in these animals associated with increasing dose, which would also be expected to be present in the urothelium. This would likely lead to a slight widening between urothelial cells even prior to death, and upon death, the autolytic process would be greatly enhanced by the fact that the cells have already separated to some extent.

This process has not been adequately evaluated. However, reanalysis of a study of sodium saccharin administered in the food to monkeys beginning at birth and continuing for up to 24 years (Takayama *et al.*, 1998) provides some useful information (unpublished observations, Cohen). Half of the monkeys in the study were processed routinely, i.e., the bladder was put in formalin fixative 20–30 min after the death of the animal. Vacuoles were present in the urothelium of the bladder of each of these monkeys. In contrast, the urinary bladders from the other monkeys in the study were inflated with fixative immediately upon the death of the animal; vacuoles were not present in the urothelium of any of these bladders.

This effect could be evaluated with PPARγ or combined agonists by comparing control and high-dose animals, having their bladders (ureters, kidney pelves) removed at necropsy and having part of the tissue immediately placed in fixative and then other portions of the tissue placed in fixative after varying lengths of time up to 30 min or more. Evaluation of the effect of systemic edema requires the use of some other agent (not a PPAR agonist) that produces edema and comparing the results to monkeys administered the PPARγ agonists.

There has also been mention of a hyperplastic response in the monkey urothelium (El-Hage, 2005). Although this needs to be critically evaluated, a significant difficulty arises in how one defines hyperplasia. For monkey and human bladders, the normal urinary bladder urothelium is considered normal up to seven cells in thickness (Murphy *et al.*, 2004; Takayama *et al.*, 1998, 1999). This is in contrast to the rodent, where 3–4 cells are considered normal. However, in the monkey and in the human, there is marked variation in the number of cells in the urothelium depending upon the history of the individual, where in the urinary bladder the specimen is taken from, and the amount of urine that has been handled by the individual prior to the obtainment of the urothelial sample for histology. Although it has not been carefully evaluated, it is generally accepted among urologic pathologists, both veterinary and human, that a major influence on the number of cell layers in the bladder urothelium is the amount of urine that is processed (Murphy *et al.*, 2004). This is somehow related to the amount the bladder has been distended, possibly due to generation of growth factors that can occur with stretching (Zhou *et al.*, 2005). Since the PPARγ and combined agonists produce fluid changes in monkeys like in rodents and humans, the urinary volume of the monkeys is likely to be greater as the dose of the agonist being administered is increased. This would be associated with increased urinary output and likely would be associated with an increase in the number of cell layers seen in the bladder urothelium, but would be diagnostically considered within the range of normal, not hyperplastic. Depending on the timing of collection of the specimens, the increase in number of cell layers might even involve a transient increase in the rate of urothelial cell proliferation (i.e., an increase in labeling index).

SUMMARY AND CONCLUSIONS

PPARγ and combined agonists have been shown to produce urothelial tumors in rats and a suggestion of urothelial changes in the monkey (and possibly the dog). These effects are either due to a direct effect on PPARγ in the urothelium or a pharmacologically based indirect mechanism involving fluid changes in the animal leading to alterations in urine composition. Such urine compositional changes produce urinary solids which are cytotoxic to the urothelium, resulting in regenerative proliferation and, ultimately, tumors. This is expected to be greater in male than in female rats, and greater in rats than in mice. It is much less likely to occur in primates, including humans.

An indirect pharmacologic mode of action produced by PPAR agonists is not unique for the urothelium. Such an indirect effect has also been seen with PPARα agonists in production of rat pancreatic acinar cell tumors and, to some extent, rat testicular Leydig cell neoplasms. These neoplasms also do not appear to be relevant to humans (Klaunig *et al.*, 2003). A variety of other indirect mechanisms have been identified with other classes of chemicals operating through other modes of action.

A direct effect on urothelial PPARγ receptors as the cause of the carcinogenic response is highly unlikely for a variety of reasons, most notably the fact that the biologic effect of these agonists on the urothelium is to inhibit proliferation rather than to increase the rate of proliferation expected for a carcinogen.

The direct and indirect hypotheses are readily testable. However, based on theoretical and practical considerations, the indirect mode of action at the present time appears more likely. These agents have been in the clinic for more than 10 years, and although no individual patient has been treated that long with any one of these agents, we are nearing a time when a signal would be expected to be detectable if these agents are bladder carcinogens in humans. Such a signal has not been reported, despite some relatively careful evaluations of human populations. Also, clinical trials have not revealed any evidence of urinary calculus formation or urothelial toxicity by PPARγ or combined agonists. Even if calculi were to occur, they do not pose a significant carcinogenic risk in humans.

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