JPET Fast Forward. Published on October 17, 2003 as DOI:10.1124/jpet.103.057208

JPET # 57208

Suppression of Cytochrome P450 2E1 Promoter Activity by Interferon-γ and Loss of Response Due to the -71G>T Nucleotide Polymorphism of the CYP2E1*7B Allele

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Running title: Interferon-γ suppresses *Cytochrome P4502E1* Promoter Activity

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Text pages: 14

Table: 1

Figures: 2

References: 38

Abstract: 145 words

Introduction: 675 words

Discussion: 827 words

Abbreviations: cytochrome P4502E1; CYP2E1, interferon-γ; IFN-γ, gamma-interferon activation sequence; GAS.

Section assignment: Cellular & Molecular or Inflammation & Immunopharmacology

Keywords: *CYP2E1*, polymorphism, interferon-γ and gene regulation.

Abstract

The *CYP2E1*7B* allele is defined by 2 nucleotide sequence polymorphisms -71G>T and -333T>A. The *CYP2E1* promoter sequence flanking the -71G nucleotide is consistent with a γ-interferon activated sequence (GAS). Inflammation and IFN-γ suppress expression of CYP2E1 *in vivo* however the exact mechanism is not known. The objectives of this study were to determine if the *CYP2E1* promoter is regulated by IFN-γ and to examine the influence of the nucleotide substitutions on this function. Treatment of HepG2 cells with IFN-γ, following transient transfection with a luciferase reporter gene bearing the native *CYP2E1* (-71G) promoter sequence resulted, in a dose-dependent reduction of luciferase activity. In contrast, no suppression was observed in cells transfected with the **7B* allele promoter (-333A and -71T) nor a *CYP2E1* plasmid containing only the -71T polymorphism. These data indicate that IFN-γ suppresses native *CYP2E1* promoter activity and that the -71G is critical for this response.

Cytochrome P450 2E1 (CYP2E1) is a major catalyst responsible for the metabolism and bioactivation of many low, molecular-weight carcinogens and potentially toxic chemicals that include ethanol, nitrosamines, halogenated alkanes, and vinyl chloride (Gonzalez and Gelboin 1994; Guengerich et al., 1991; Uziel et al., 1992; Whysner et al., 1996). These compounds are converted by CYP2E1 into inert polar metabolites or bio-activated into reactive intermediates that are able to initiate aseptic inflammation, toxicity and DNA mutations.

CYP2E1 expression can be induced by ethanol, acetone, and other small compounds that result in enhanced hepatic damage (Lindros et al.; 1990; Tsutsumi et al., 1993; Neuman et al., 1999). In contrast, CYP2E1-null mice are resistant to the CYP2E1 bioactivation-dependent toxicity of a number of substrates for this enzyme (Valentine et al., 1996; Zaher et al., 1998). These data suggest that the level of CYP2E1 expression *in vivo* is related to drug or environmental-induced liver toxicity. Thus, there is the liklihood that inter-individual differences in the expression and function of CYP2E1 may result in differential challenge to the cellular detoxification mechanisms. For this reason, many studies have focused on understanding the mechanisms that control CYP2E1 expression, with the objective of gaining insight into mechanisms of toxicity and opportunities for protective intervention.

Human CYP2E1 mRNA and protein exhibit wide inter-individual variability (Peter et al., 1990; Lucas et al., 1992). We and others have proposed that genetic polymorphisms of the gene encoding *CYP2E1* (Tanaka 1999; Antonino-Green et al., 2000) could contribute to such variability. Many *CYP2E1* alleles have been identified,

JPET Fast Forward. Published on October 17, 2003 as DOI: 10.1124/jpet.103.057208
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including *CYP2E1*1A* which refers to the native gene (Oscarson, 2001; Tanaka 1999) (www.imm.ki.se/CYPalleles.htm). Four alleles of *CYP2E1*; *CYP2E1*1D*, *CYP2E1*5B*, *CYP2E1*6* and *CYP2E1*7B*, have been associated with increased susceptibility to cancer or altered transcriptional expression (Howard et al., 2003, McCarver et al., 1998, Hayashi et al., 1991, Huang et al., 1997, Uematsu et al., 1991, Watanabe et al., 1994, Fairbrother et al., 1998). Of these alleles, most attention has focused on the *CYP2E1*1D* and *CYP2E1*5B* alleles. There is now compelling evidence that the *CYP2E1*1D* allele results in meaningful phenotypic differences in inducible CYP2E1 expression (McCarver et al., 1998), as well as contributing to alcohol and nicotine dependence (Howard et al., 2003). In contrast, with the exception one study which found no association between the CYP2E1*7B allele and alcohol-induced pancreatic cancer (Yang et al., 2001), the potential for phenotypic consequences of the CYP2E1*7B allele have not been studied in detail. The nucleotide sequence variants of *CYP2E1*7B* (-71G>T; -333T>A) are in the 5' transcriptional regulatory region of this gene. The functional significance of polymorphisms found in the *CYP2E1*7B* allele have been studied in the context of chimeric luciferase reporter genes transiently expressed in HepG2 cells. A 1.8-fold increase in luciferase activity was found for the **7B* chimera, including -71G>T and -333T>A substitutions, compared with the native sequence (Fairbrother et al., 1998). However, no consistent changes in CYP2E1 activity were observed in human livers of individuals with the heterozygous *CYP2E1*1/CYP2E1*7B* genotype. Thus, functional importance of a this relatively common (frequency; 0.052 ± 0.028) human *CYP2E1* allele remains in question (Fairbrother et al., 1998).

We discovered that the -71G>T nucleotide polymorphism is located within a consensus sequence motif commonly known as a GAS (Gamma-interferon Activation Sequence) element Table 1. GAS elements are short stretches of DNA with the general structure $TTCN_{2-4}GAA$ originally defined as a requirement for the rapid transcriptional induction or suppression of genes in response to IFN-γ. These structures are now known to be involved in the transcriptional regulation of a number genes via a variety of cytokines, inflammatory mediators and peptide hormones (Decker et al., 1997). Cytochrome P4502E1 activity in mice, rats, and humans is suppressed by IFN-γ (Morgan 1993; Morgan 2001). Interferon-γ suppresses the CYP2E1 mRNA content of adult human hepatocytes (Abdel-Razzak et al., 1993) and *in vivo* production of interferon α/β in rats down regulates CYP2E1 expression that is accompanied by a corresponding decrease in CYP2E1 mRNA (Anari et al., 1995). The objectives of these studies were to determine if the *CYP2E1* promoter activity is regulated by IFN-γ, and to determine the influence of the CYP2E1*7B allele nucleotide substitutions on this function.

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Materials and methods

Cell culture and IFN-γ treatment

Human hepatoma (HepG2) cells were cultured in Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% non-essential amino acids (Mediatech, InC. Herndon, VA). For treatment with recombinant human IFN-γ (Roche Molecular Biochemicals. Indianapolis, IN), cells were grown to 70% confluency, and allowed to stay in serum-free medium for 24-hours prior to treatment. The experimental cells were treated by adding 500 unit/ml of IFN-γ directly to the culture medium. Conversely, the control cells were run in parallel without IFN-γ. Cultured cells were used in the preparation of nuclear extracts and in transient transfection studies.

CYP2E1 chimeric luciferase reporter gene bearing -71G>T substitution.

To study the functional significance of *CYP2E1* -71G>T substitution, a *CYP2E1* chimeric luciferase reporter gene, bearing -71G>T substitution only, (*CYP2E1*$_{-71G>T}$), was synthesized from a plasmid carrying a native *CYP2E1* sequence (*CYP2E1*$_{native}$) by using the GeneEditor™ *in vitro* site-directed mutagenesis system (Promega, Madison WI) (*CYP2E1*$_{native}$ and the *CYP2E1* plasmid containing the **7B* allele substitutions, *CYP2E1* $_{*7B,}$ were generously provided by Dr. A.K. Daly at University Newcastle Upon Tyne, UK) (Fairbrother et al., 1998). The *CYP2E1* gene promotor sequence from –585 to –33 containing the native –71G was inserted into a pGL2-enhancer vector upstream of the luciferase reporter gene. Note that the nucleotide sequence positions are relative to the A of the ATG-translation initiation codon as recommended by den Dunnen et al. 2001. In the original manuscript (Fairbrother et al. 1998), nucleotide sequence numbering was -

549 to +3 relative to the transcription initiation site. By denaturing the *CYP2E1*$_{native}$ plasmid with alkaline, the phosphorylated *CYP2E1* primer containing a T_{-71}, along with a phosphorylated primer that alters the ampicillin resistance gene (provided by the GeneEditor^{TM} system), were annealed. Mutations were then introduced by the aid of T4 DNA polymerase and T4 DNA ligase. Bacteria containing the -71G>T substitution (*CYP2E1*$_{-71G>T}$) were then selected by antibiotic resistance. The sequence of the plasmids of *CYP2E1*$_{native}$, *CYP2E1* $_{*7B}$ and *CYP2E1*$_{-71G>T}$ were confirmed by sequencing.

Transfection of HepG2 cells and IFN-γ treatment

HepG2 cells were seeded in six-well tissue culture plates at 2 x 10^5 cells/well and cultured in 2 ml of medium supplemented with 10% FBS until 50% confluent. For each set of wells, 2 μg of each experimental plasmid (*CYP2E1*$_{native}$, *CYP2E1*$_{*7B}$ and *CYP2E1*$_{-71G>T}$ respectively) and 2 μg of pSV-β-galactosidase control plasmid (Promega, Madison, WI) were diluted into 100 μl serum-free medium and then mixed with 100 μl of serum-free medium containing 0.3 μg/μl Lipofectamine Reagent (GibCo BRL, Rockville, MD). The DNA-liposome complexes were allowed to form at room temperature for 30-minutes. Following transfection, the cells were washed twice with serum-free medium then cultured in 800 μl of serum-free medium. After 16-hours, the medium was replaced with the original culture medium and the cells were allowed to recover for 24-hours. The cells were then maintained in serum-free medium for another 24-hours before the treatment. The experimental cells were treated by adding IFN-γ directly to the serum-free medium (100 - 500 unit/ml) whereas the control cells were run in parallel without IFN-γ. The cells were lysed after 24-hours incubation by reporter lysis buffer (Promega, Madison, WI). The cytoplasm was collected and protein concentration was measured.

Luciferase activity was measured with a manual luminometer, Lumat LB9507 (EG&G Wallac, Gaithersberg MD). The β-galactosidase activity was measured using a β-gal assay kit (Promega, Madison, WI). The luciferase activity for each experiment was normalized for β-galactosidase activity and total protein concentration. Dose-response studies were performed in triplicate for each concentration of IFN-γ tested. Differential response between native and variant reporter genes was tested in three independent experiments, with a minimum of 4 wells per experimental group. Statistical analysis was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA) by one-way analysis of variance (ANOVA) followed by the appropriate tests for pairwise differences (see Figure legends).

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Results

Sequence comparison of CYP2E1 proximal promoter and identification of a γ-interferon activation sequence (GAS)

The nucleic acid sequence of the human *CYP2E1* gene proximal promoter was screened for the presence of potential transcriptional regulatory domains (http://transfac.gbf.de/TRANSFAC) (Wingender et al 2000). We did not identify specific regulatory sequences which included the -333T> A substitution as a critical site of interaction. In contrast, we found that the nucleotide sequence which includes the -71G>T substitution was consistent with the structure of GAS (Gamma-interferon Activation Sequence) elements which have been identified as essential for IFN-γ or other cytokine dependent regulation of transcription (Strehlow et al. 1993), (Table 1).

Differential regulatory response of CYP2E1$_{native}$ vs *CYP2E1 $_{*7B}$ and CYP2E1$_{-71G}$ to IFN-γ*

To test the ability of IFN-γ to alter the transcriptional expression of the human *CYP2E1* promoter, we transiently transfected HepG2 cells with a construct containing 552 bp of *CYP2E1* gene promoter sequence driving transcription of a luciferase reporter gene. As shown in Figure 1, IFN-γ treatment of cells transfected with the *CYP2E1$_{native}$* plasmid resulted in a dose-dependent decrease in luciferase activity, compared with untreated cells (Tukey test; $p < 0.05$).

To study the response of the CYP2E1*7B promoter sequence to IFN-γ, HepG2 cells were transfected with the *CYP2E1$_{*7B}$* reporter gene that is identical in size and sequence to the native reporter gene with the exception of the two nucleotide substitutions (-71G>T and -333T>A). As shown in figure 2, this reporter gene was

entirely unresponsive to the highest concentration of IFN-γ tested (500 U/ml). To determine whether the -71G>T substitution was sufficient to account for this loss of function, a reporter gene was constructed from the *CYP2E1*$_{native}$ plasmid to include only the -71G>T substitution (*CYP2E1*$_{T\text{-}71}$). As was true for the *CYP2E1*$_{*7B}$ reporter gene, expression of the *CYP2E1*$_{T\text{-}71}$ construct was also unsuppressed by IFN-γ, demonstrating that this single nucleotide polymorphism is sufficient to account for the loss of IFN-γ mediated suppression of the CYP2E1*7B promoter activity. Basal expression of these reporter genes was indistinguishable and differences in expression were observed only in response to IFN-γ challenge.

Discussion

We have discovered a previously unrecognized regulatory domain within the human *CYP2E1* gene that is structurally consistent with a gamma-interferon activation sequence (GAS), and includes a naturally occurring nucleotide sequence polymorphism. This nucleotide sequence, (-77 through -69) of the human *CYP2E1* gene, is located between the basal transcription element (BTE) and the TATA box. Comparison of the rat, human, and mouse CYP2E1 genes reveals that the general GAS structure (TTCN $_{2-4}$ GAA) is present in each species. The rat and mouse structures include one additional nucleotide within the central variable region of the GAS motif, (Table 1). The difference in spacing between the flanking palindromic sequences of GAS elements has not been shown to significantly alter the function of these regulatory elements (Decker et al. 1997), suggesting that the regulatory role of this element is conserved between species.

The putative GAS element of the human *CYP2E1* is virtually identical to the GAS element of the human IFP53 gene (Strehlow et al. 1993). This structure is responsible for the IFN-γ-dependent regulation of IFP53 transcription. In the present study, we demonstrate that transcription driven by the human *CYP2E1* proximal promoter is suppressed by IFN-γ and that the single nucleotide sequence polymorphism (-71G>T) is sufficient to account for the loss of IFN-γ response of the proximal *CYP2E1*7B* promoter.

Many previous studies have demonstrated that cytochromes P450 are down regulated by mediators of the inflammatory response (reviewed in Morgan et al. 2002). It has been observed in rats that endotoxin (Morgan, 1997) and polyinosinic acid-

polycytidylic acid which induces endogenous production of interferons (Cribb et al., 1994), suppresses CYP2E1 mRNA and protein. Furthermore, IFN-γ can suppress CYP2E1 mRNA in primary culture of human hepatocytes (Abdel-Razzak et al., 1993) and protein expression in mice (Anari et al., 1995). These data all suggest that inflammatory mediators could regulate *CYP2E1* gene expression at the transcriptional level. The major factor governing constitutive expression of *CYP2E1* is reported to be hepatocyte nuclear factor 1α (HNF 1α) (Umeno and Gonzalez, 1990) and changes in HNF 1α binding to the proximal promoter of *CYP2E1* has been suggested as one potential mechanism to account for suppressed CYP2E1 expression by inflammatory cytokines (Roe et al., 2001 ; Hakkola et al., 2003). However, decreased binding activity of HNF 1α did not fully and consistently account for inflammatory cytokine mediated suppression of the rat or mouse *CYP2E1* genes (Roe et al. 2001; Hakkola et al. 2003). The putative GAS element described here is in close proximity but does not overlap with the HNF 1α binding site. Although this does not preclude interaction between these regulatory domains, our data does argue against an exclusive role for altered HNF 1α binding as the mechanism to account for suppressed transcriptional activity. There is minimal (Fairbrother et al. 1998), if any, influence of the -71G>T substitution on the constitutive activity of the human *CYP2E1* proximal promoter. However, this single nucleotide polymorphism was sufficient to abolish IFN-γ dependent suppression of promoter function, suggesting that this nucleotide position directly alters binding of a transcription factor that does not control constitutive expression. Thus, as proposed by Hakkola et al., there are likely to be a variety of transcription factors involved in the complex transcriptional regulation of the *CYP2E1* gene. With the exception of one study

that failed to demonstrate any role for the *CYP2E1*7B* allele in alcoholic pancreatitis (Yang et al., 2001), the relationship between this allele and human disease has otherwise not been researched. We hypothesize that the human *CYP2E1*-GAS element is one part of a negative-feedback regulatory mechanism to limit the intracellular pool of CYP2E1 enzyme and thus limit the accumulation of reactive metabolic intermediates. Several substrates of this enzyme are initially bio-activated to reactive intermediates (Guengerich et al., 1991) and initiate an aseptic inflammatory response (McClain et al., 1999). Aseptic inflammation causes suppression of multiple cytochrome P450 enzymes including CYP2E1 (Siewert et al., 2000). Thus, under circumstances where reactive intermediates begin to accumulate and present an inflammatory challenge, a negative-feedback signal is generated (potentially including IFN-γ) leading to suppressed CYP2E1 enzyme synthesis and ultimately limiting the accumulation of reactive intermediates of phase I metabolism. Further, we hypothesize that the CYP2E1*7B allele could potentially increase risk and or degree of toxicity due to failure of this mechanism and the ensuing accumulation of reactive intermediates.

In conclusion, our studies are the first report of a GAS structure within the proximal promoter region of the human *CYP2E1* and the first evidence for a functional consequence of the -71G>T substitution. Further, these results support previous indications that *CYP2E1* transcription is down regulated by IFN-γ and also provide an additional molecular mechanism for this regulation. Ongoing studies in our laboratory are focusing on: identifying trans-factors which directly or indirectly interact with the GAS structure of the *CYP2E1* gene, establishing causality between accumulation of metabolic intermediates and transcriptional suppression, and in determining whether

failure of negative-feedback regulation is sufficient to alter metabolic balance in favor of increasing the toxic potential of CYP2E1 substrates.

Acknowledgements

We thank Dr. A.K. Daly, University Newcastle Upon Tyne, UK, for kindly providing several CYP2E1 reporter genes, and Rosemary Williams for her assistance in the preparation of this manuscript.

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Footnotes

This work was supported in part by NIEHS, R01 ES08953-01, and an intramural grant from the University of Louisville School of Medicine.

JPET Fast Forward. Published on October 17, 2003 as DOI: 10.1124/jpet.103.057208
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Figure 1. Dose-dependent suppression of native *CYP2E1* promoter activity by IFN-γ.

HepG2 cells were treated with increasing concentrations of IFN-γ (100 - 500 unit/ml) following transfection with $CYP2E1_{native}$ plasmid. Luciferase activities were normalized for β-galactosidase activity and lysate protein concentration. Asterisk designates values that were judged to different from the control based on pairwise multiple comparisons, Tukey Test; $p < 0.05$. [Values are means ± SD (n = 3 for each group)].

Figure 2. Polymorphism of the GAS structure is sufficient to account for loss of γ-IFN response of the *CYP2E1*7B* allele promoter. HepG2 cells were transfected with $CYP2E1_{native}$ (T -333 / G -71), $CYP2E1_{*7B}$ (A – 333 / T -71) or $CYP2E1_{T_{-71}}$ (T -333 / T -71) reporter genes and cultured for 24 hrs in the absence (solid bar) or presence (open bar) of IFN-γ (500 unit/ml). Luciferase activities were normalized for β-galactosidase activity and lysate protein concentration. Statistical analysis was performed by analysis of variance, ANOVA; $p < 0.001$. [Values are means ± SD (n = 12 for each group)].

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TABLE 1. Sequence comparison of GAS element in the promoter of CYP2E1 and other IFN-γ inducible genes.

GAS Sequence	Gene	Regulator
GGT**TTC**CGG**GAA**AGC[a]	human ICAM (Intercellular Adhesion molecule)	IFN-γ
TGT**TTC**TGA**GAA**TCT[a]	human IFP_{53} (tryptophanyl tRNA synthetase)	IFN-γ
TCC**TTC**TCA**GAA**CAC[a,b]	human *CYP2E1*	??
TCC**TTC**TCAa**GGA**TAC	rat CYP2E1	??
TCC**TTC**TCAt**GAA**CAC	mouse CYP2E1	??

[a] The consensus sequences for GAS elements are shown in bold type; [b] the -71G in the human *CYP2E1* gene is underlined. Rat CYP2E1 sequence Umeno 1988, lower case nucleotides indicate insertions found in the mouse and rat genes in comparison to the human gene.

Figure 1.

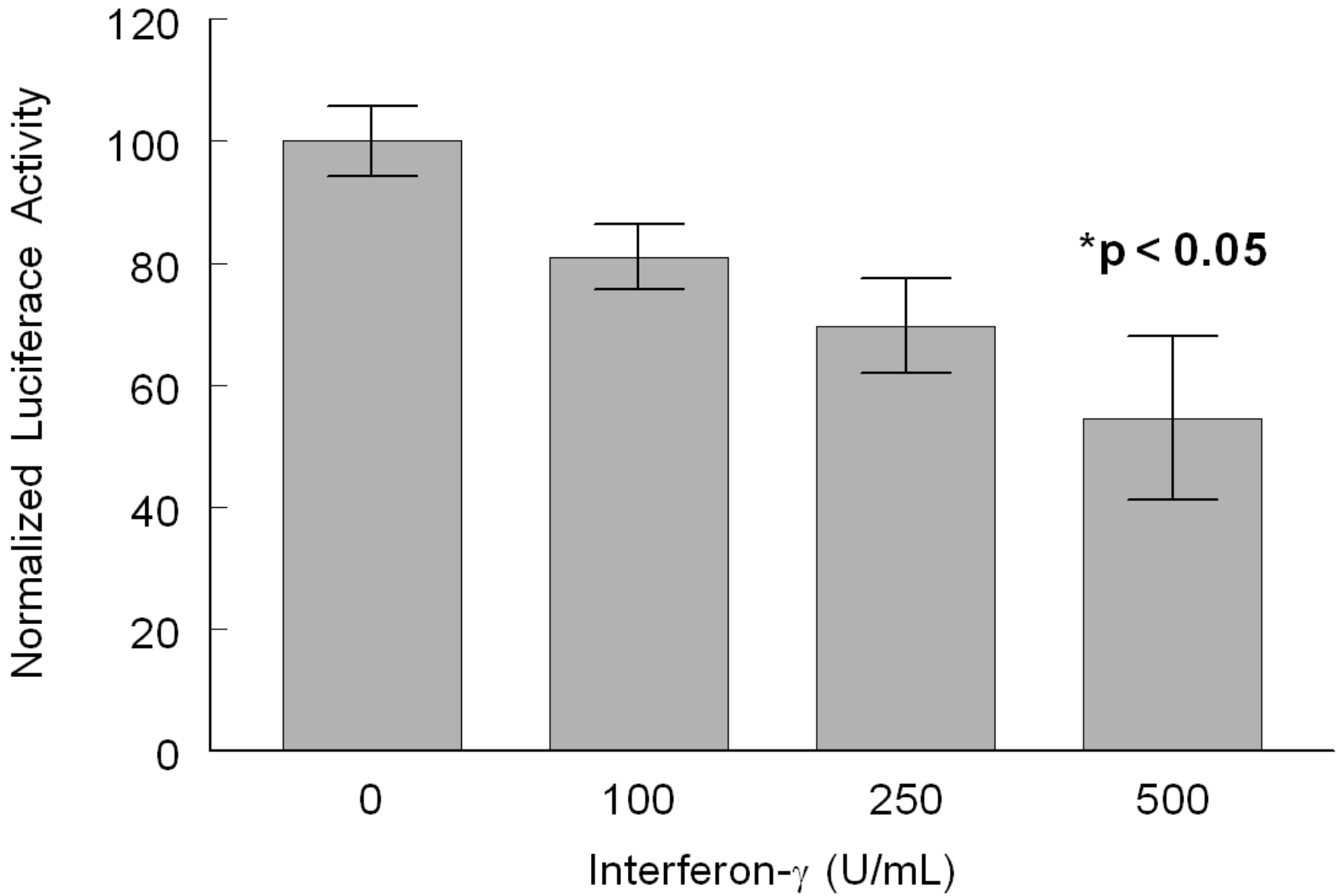

Figure 2.