5504 *Biochemistry* **2009,** *48,* 5504–5506
DOI: 10.1021/bi9006258

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

Antagonism of Human Adiponectin Receptors and Their Membrane Progesterone Receptor Paralogs by TNFα and a Ceramidase Inhibitor[†]

Brian R. Kupchak,[‡] Ibon Garitaonandia,[§] Nancy Y. Villa,[∥] Jessica L. Smith,[⊥] and Thomas J. Lyons*

Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611. [‡]Current address: University of Connecticut, Storrs, CT 06269. [§]Current address: Scripps Research Institute, La Jolla, CA 92037. [∥]Current address: University of Florida, Gainesville, FL 32611. [⊥]Current address: National Institutes of Health, Frederick, MD 21702

Received April 13, 2009; Revised Manuscript Received May 14, 2009

Downloaded by UNIV OF FLORIDA on August 14, 2009
Published on May 19, 2009 on http://pubs.acs.org | doi: 10.1021/bi9006258

ABSTRACT: The progestin and AdipoQ receptor (PAQR) family of proteins comprises three distinct structural classes, each with seemingly different agonist specificities. For example, Class I receptors, like the human adiponectin receptors (AdipoR1 and AdipoR2), sense proteins with a particular three-dimensional fold, while Class II receptors are nonclassical membrane receptors for the steroid hormone progesterone. Using a previously developed heterologous expression system to study PAQR receptor activity, we demonstrate that human PAQRs from all three classes are antagonized by both 1(*S*),2(*R*)-D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol, a ceramidase inhibitor, and TNFα, a homologue of adiponectin that functions antagonistically to both adiponectin and progesterone in human cells.

The progestin and AdipoQ receptor (PAQR)[1] family of proteins comprises several medically important hormone receptors with links to pathological conditions, including obesity, diabetes, and coronary artery disease (*1*). PAQR proteins can be grouped into three distinct classes on the basis of sequence comparisons. Class I receptors are found in nearly all eukaryotes. The two best-characterized human Class I receptors are the adiponectin receptors, AdipoR1 (PAQR1) and AdipoR2 (PAQR2), which sense adiponectin, an adipose-derived hormone in the C1q/TNF superfamily of animal proteins (*1*). A third Class I PAQR (Izh2p) from the fungus *Saccharomyces cerevisiae* senses plant proteins in the PR-5 defensin superfamily whose three-dimensional fold is nearly identical to the β-sandwich of the C1q/TNF superfamily (*2*, *3*). Consequently, it appears as though the unifying feature of Class I PAQRs may be the ability to bind proteins with a specific β-sandwich fold.

On the other hand, the Class II PAQRs, including mPRα (PAQR7), mPRβ (PAQR8), and mPRγ (PAQR5), diverged from Class I receptors after the evolution of metazoans. Intriguingly, the steroid hormone progesterone agonizes these receptors (*4*, *5*), and it is unclear how this functionality evolved from Class I β-sandwich receptors. Finally, there is the enigma of the Class III PAQRs, which have the deepest evolutionary roots but no known agonist. Not only do all metazoans have at least one Class III protein, they are widely, but not universally, dispersed in protozoan and eubacterial proteomes (*6*). Because Class III receptors predate Class I receptors, one might predict that their most recent common ancestor may have sensed a β-sandwich-like protein.

There is considerable pharmaceutical interest in identifying novel molecules that can agonize human PAQR receptors. In particular, because adiponectin is anti-diabetic, there is considerable interest in finding agonists for AdipoR1 and AdipoR2 that might be useful pharmaceuticals for the treatment of obesity or type II diabetes (*1*). However, recent studies suggest that these two receptors, while sensing the same agonist, may have opposing physiological roles (*7*). Consequently, the search for molecules that target PAQRs should be expanded to look for antagonists as well as agonists.

We have developed a yeast-based assay system that can be used to study the functionality of human PAQRs. The details of this assay have been extensively described elsewhere (*4*, *8*, *9*) and are summarized in the Supporting Information. In brief, the assay is based on the fact that PAQR receptors in *S. cerevisiae* (named Izh1p, Izh2p, Izh3p, and Izh4p) activate an intracellular signaling cascade that negatively controls the expression of a gene called *FET3*. We generated a β-galactosidase-based *FET3* promoter−reporter construct whose activity is inversely proportional to the activity of the expressed PAQR receptor.

Using this assay, we demonstrated functional expression of AdipoR1, AdipoR2, mPRγ, mPRα, and mPRβ. In addition, we discovered that the human Class I receptor, PAQR3, is activated by adiponectin and renamed it AdipoR3 (*9*). Moreover, we discovered that the two remaining human Class II receptors, PAQR6 and PAQR9, are agonized by progesterone and renamed them mPRδ and mPRε, respectively (*4*).

[†]This research was funded by the National Institutes of Health (Grant R21DK074812 to T.J.L.) and by the University of Florida Department of Chemistry.

*To whom correspondence should be addressed: Foundation for Applied Molecular Evolution, P.O. Box 13174, Gainesville, FL 32604. E-mail: tlyons@ffame.org. Telephone: (352) 271-7005. Fax: (352) 271-7076.

[1]Abbreviations: PAQR, progestin and AdipoQ receptor; TNF, tumor necrosis factor; MAPP, 1(*S*),2(*R*)-D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol; AdipoR, adiponectin receptor; mPR, membrane progestin receptor; ID_{50}, 50% maximum inhibitory dose; ED_{50}, 50% maximum effective dose.

 Published on Web 05/19/2009 © 2009 American Chemical Society

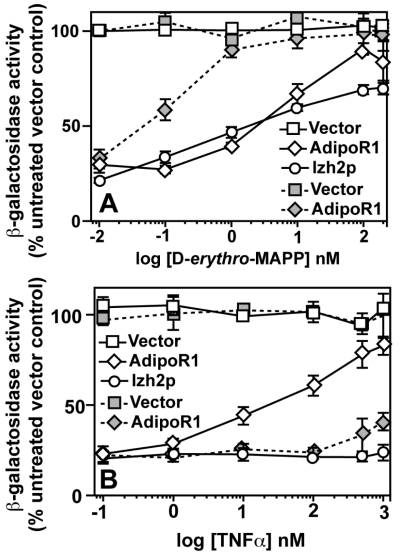

FIGURE 1: (A) MAPP inhibits basal signaling of maximally expressed AdipoR1 and Izh2p (white symbols) as well as AdipoR1 when activated by 100 pM adiponectin at lower expression levels (gray symbols). (B) TNFα inhibits basal and adiponectin-dependent signaling of AdipoR1 but not basal signaling of Izh2p. The legend is the same as that for panel A.

We made another intriguing discovery with our yeast-based assay. For the yeast Izh2p and several human receptors (AdipoR1, PAQR3, PAQR4, mPRγ, mPRβ, and PAQR11), agonist is not absolutely required to activate the downstream signaling pathway (*2*, *4*, *8*, *9*). In these cases, maximal overexpression is sufficient to constitutively activate the pathway, indicating that some receptors in this family possess significant basal signaling capability. While we are unsure why some receptors possess such high basal activity, this is an important discovery because it allows us to use our assay to screen for different types of antagonists, including both competitive antagonists and inverse agonists, which are a special type of antagonist that inhibits basal signaling.

We recently published data demonstrating that fungal PAQRs produce a sphingoid base second messenger in yeast by activating an endogenous ceramidase enzymatic activity (*2*). We also demonstrated that MAPP, a potent ceramidase inhibitor, strongly inhibits both the basal and agonist-inducible signaling capability of the endogenous yeast PAQR, Izh2p. In Figure 1A, Table 1, and the figure in the Supporting Information, we show that MAPP also inhibits the basal signaling of human Class I (AdipoR1, PAQR3, and PAQR4), Class II (mPRγ and mPRβ) and Class III (PAQR11) receptors. Moreover, MAPP inhibits the agonist-inducible signaling of AdipoR1, AdipoR2, and mPRγ. The generalized inhibitory effect of MAPP on yeast and human PAQRs is not surprising considering the strong sequence similarity between PAQRs and proteins in the alkaline ceramidase family (*2*). The fact that MAPP inhibits both the basal and activated signaling of PAQRs indicates that this molecule acts as an inverse agonist, potentially by inhibiting the mechanism of signal transduction. However, it must be stated that we cannot yet rule out the possibility that MAPP acts on the signaling pathway downstream of the receptor rather than directly on the receptor.

We also report the remarkable finding that human proteins in the PAQR family, including AdipoR1 and AdipoR2, are unified by antagonism by the inflammatory cytokine, tumor necrosis factor α (TNFα), itself a β-sandwich protein in the C1q/TNF superfamily. The C1q/TNF superfamily has two basic subfamilies: C1q-like proteins, including adiponectin, and TNF-like proteins, including TNFα (*10*). A quick perusal of the literature reveals that TNFα and adiponectin often exert opposite or antagonistic effects in human cells. For example, while there are low levels of adiponectin in obesity, there are high levels of TNFα. More importantly, while adiponectin is anti-diabetic and anti-inflammatory, TNFα is pro-diabetic and pro-inflammatory (*11*).

Table 1: Antagonism of Human PAQR Signaling by MAPP and TNFα

receptor	ID_{50} for MAPP (nM)	ID_{50} for TNFα (nM)
PAQR1 (AdipoR1)[a]	5.2 ± 1.6	53.1 ± 1.5
PAQR1 (AdipoR1)[b]	0.1 ± 1.0	nd[e]
PAQR2 (AdipoR2)[a]	17.0 ± 1.2	nd[e]
PAQR2 (AdipoR2)[b]	3.0 ± 1.3	nd[e]
PAQR3 (AdipoR3)[a]	12.6 ± 1.3	2.1 ± 1.5
PAQR4[a]	18.2 ± 1.7	4.8 ± 1.5
PAQR5 (mPRγ)[a]	5.5 ± 2.8	3.3 ± 2.4
PAQR5 (mPRγ)[b]	3.0 ± 1.8	nd[e]
PAQR8 (mPRβ)[a]	6.5 ± 1.5	3.9 ± 1.2
PAQR11 (MMD1)[a]	89.1 ± 3.5	2.8 ± 1.8

	agonist ED_{50}	
receptor	without TNFα	with TNFα
PAQR1 (AdipoR1)[c]	0.7 ± 1.2	3.5 ± 1.3
PAQR2 (AdipoR2)[c]	2.4 ± 1.4	10.5 ± 1.4
PAQR3 (AdipoR3)[c]	20.1 ± 2.8	73.5 ± 1.2
PAQR5 (mPRγ)[d]	5.6 ± 1.2	15.1 ± 2.1
PAQR6 (mPRδ)[d]	7.2 ± 1.2	28.5 ± 1.5
PAQR8 (mPRβ)[d]	3.6 ± 2.2	12.1 ± 1.6

[a] Maximal receptor expression, no agonist added. [b] Low level of receptor expression, agonist added: 100 pM adiponectin for AdipoR1 and AdipoR2 and 100 nM progesterone for mPRγ. [c] Adiponectin as the agonist, values in picomolar. Low level of receptor expression, with 100 nM TNFα. [d] Progesterone as the agonist, values in nanomolar. Low level of receptor expression, with 100 nM TNFα. [e] Not determined.

Given that TNFα opposes adiponectin and that the two proteins belong to the same structural family, we postulated that TNFα might antagonize the effects of adiponectin on adiponectin receptors. Table 1, Figure 1B, and the figure in the Supporting Information show that this is indeed the case for AdipoR1. In addition, TNFα acts as an inverse agonist for AdipoR1, shutting off its basal signaling (Figure 1B). More importantly, TNFα does not affect signal transduction in yeast when the endogenous Izh2p receptor is used to activate the pathway. These results clearly demonstrate that TNFα does not act on the yeast signaling pathway downstream of the receptors or else it would have a similar effect on Izh2p. The possibility that TNFα is a more general antagonist of human PAQRs is supported by data listed in Table 1, which demonstrate TNFα-dependent inhibition of basal signaling of PAQR3, PAQR4, mPRγ, mPRβ, and PAQR11. Moreover, TNFα antagonizes the effect of agonists on AdipoR1, AdipoR2, PAQR3, mPRγ, mPRδ, and mPRβ. The only receptors that do not seem to be affected by TNFα are mPRα and PAQR9.

It is critical to emphasize that these results show an effect of TNFα in yeast cells expressing human PAQRs, not on human cells. The yeast genome does not encode C1q/TNF family members or homologues of the classical TNFα receptors (TNFR) (*12*). Hence, the effect of TNFα in our system must be independent of the known mechanisms of sensing TNFα since these systems are absent in yeast. Moreover, since TNFα has no effect on yeast cells carrying the empty expression vector or

Downloaded by UNIV OF FLORIDA on August 14, 2009
Published on May 19, 2009 on http://pubs.acs.org | doi: 10.1021/bi9006258

overexpressing an endogenous yeast PAQR, the possibility that a yeast protein mediates the effects of TNFα rather than the expressed human PAQR is very remote. The simplest explanation for our extraordinary finding is that TNFα functions as both a general competitive antagonist and an inverse agonist for human PAQRs. Of course, we will need to show direct binding of TNFα to the PAQRs to confirm this model, although it must be noted that direct binding of agonist to receptor has not actually been demonstrated for any PAQR, including AdipoR1. This is mainly due to the fact that no group has yet been able to purify any PAQR receptor for in vitro study.

The physiological importance of TNFα antagonism of human PAQRs is unknown. TNFα exerts maximal effects on heterologously expressed human PAQRs in the low nanomolar to midnanomolar range, concentrations that are significantly higher than steady state circulating levels of this cytokine in human plasma. Of course, it is possible that the pharmacodynamics of these receptors is fundamentally altered by heterologous expression and that our ID_{50} values are not relevant to the native system. On the other hand, temporal and spatial spikes in TNFα concentrations can be many orders of magnitude higher than the steady state adjacent to sites of inflammation (*13*), making our discovery potentially relevant under pathological conditions. Even more intriguing is the possibility that the PAQR receptors are promiscuous with respect to other members of the C1q/TNF family. Since the human genome encodes 32 C1q-like proteins and 19 TNF-like proteins (*10*, *14*), it will be interesting to explore the possibility that these proteins represent physiologically relevant PAQR agonists or antagonists.

Clearly, more experiments must be done to study the exact mechanisms by which TNFα and MAPP inhibit PAQR-dependent signaling in yeast. Moreover, it will be critical to confirm these findings in human cells. However, it is important to convey these findings immediately because they may shed light on many unexplained physiological phenomena pertaining to adiponectin, progesterone, and TNFα. For example, while it is well-known that TNFα opposes the effects of adiponectin on cells (*15*), a recent study also showed that TNFα inhibits the effects of progesterone in rat ovary cells (*16*). Hence, there is already evidence that these findings are more than a mere artifact of the in vitro assay.

It is known that sphingolipids are involved in both adiponectin and progesterone signaling (*17*, *18*). Moreover, ceramide is a known second messenger for TNFα signaling (*19*), although this relationship is generally attributed to the effect of TNFα on sphingomyelinase expression. For the first time, we can present a reasonable unified mechanism that can explain these disparate observations. Eukaryotic cells possess a regulatory module called the ceramide rheostat (*20*) that governs critical processes such as proliferation, apoptosis, and differentiation. The rheostat keeps a homeostatic balance in the relative ratios of ceramides and sphingoid bases. Since PAQRs stimulate the degradation of ceramides in yeast to produce sphingoid bases (*2*), they are in a unique position to function as regulators of this rheostat and any inverse agonist or antagonist of the PAQRs, such as MAPP or TNFα, will result in ceramide accumulation. It is intriguing to speculate that PAQR receptors function as a fulcrum in human cells for the ceramide rheostat. When they are agonized, perhaps by C1q-like proteins or progesterone, they tip the balance toward sphingoid base. When they are antagonized, perhaps by TNF-like proteins, they tip the balance toward ceramide.

SUPPORTING INFORMATION AVAILABLE

Detailed protocol and supplemental figure. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Yamauchi, T., and Kadowaki, T. (2008) *Int. J. Obes. 32* (S7), S13–S18.
2. Villa, N. Y., Kupchak, B. R., Garitaonandia, I., Smith, J. L., Alonso, E., Alford, C., Cowart, L. A., Hannun, Y. A., and Lyons, T. J. (2009) *Mol. Pharmacol. 75*, 866–875.
3. Narasimhan, M. L., Coca, M. A., Jin, J., Yamauchi, T., Ito, Y., Kadowaki, T., Kim, K. K., Pardo, J. M., Damsz, B., Hasegawa, P. M., Yun, D. J., and Bressan, R. A. (2005) *Mol. Cell 17*, 171–180.
4. Smith, J. L., Kupchak, B. R., Garitaonandia, I., Hoang, L. K., Maina, A. S., Regalla, L. M., and Lyons, T. J. (2008) *Steroids 73*, 1160–1173.
5. Zhu, Y., Bond, J., and Thomas, P. (2003) *Proc. Natl. Acad. Sci. U.S. A. 100*, 2237–2242.
6. Tang, Y. T., Hu, T., Arterburn, M., Boyle, B., Bright, J. M., Emtage, P. C., and Funk, W. D. (2005) *J. Mol. Evol. 61*, 372–380.
7. Bjursell, M., Ahnmark, A., Bohlooly, Y. M., William-Olsson, L., Rhedin, M., Peng, X. R., Ploj, K., Gerdin, A. K., Arnerup, G., Elmgren, A., Berg, A. L., Oscarsson, J., and Linden, D. (2007) *Diabetes 56*, 583–593.
8. Kupchak, B. R., Garitaonandia, I., Villa, N. Y., Mullen, M. B., Weaver, M. G., Regalla, L. M., Kendall, E. A., and Lyons, T. J. (2007) *Biochim. Biophys. Acta 1773*, 1124–1132.
9. Garitaonandia, I., Smith, J. L., Kupchak, B. R., and Lyons, T. J. (2009) *J. Recept. Signal Transduction 29*, 67–73.
10. Kishore, U., Gaboriaud, C., Waters, P., Shrive, A. K., Greenhough, T. J., Reid, K. B., Sim, R. B., and Arlaud, G. J. (2004) *Trends Immunol. 25*, 551–561.
11. Forsythe, L. K., Wallace, J. M., and Livingstone, M. B. (2008) *Nutr. Res. Rev. 21*, 117–133.
12. Collette, Y., Gilles, A., Pontarotti, P., and Olive, D. (2003) *Trends Immunol. 24*, 387–394.
13. Gohel, M. S., Windhaber, R. A., Tarlton, J. F., Whyman, M. R., and Poskitt, K. R. (2008) *J. Vasc. Surg. 48*, 1272–1277.
14. Ghai, R., Waters, P., Roumenina, L. T., Gadjeva, M., Kojouharova, M. S., Reid, K. B., Sim, R. B., and Kishore, U. (2007) *Immunobiology 212*, 253–266.
15. Son, B. K., Akishita, M., Iijima, K., Kozaki, K., Maemura, K., Eto, M., and Ouchi, Y. (2008) *Endocrinology 149*, 1646–1653.
16. Nilsson, E. E., Stanfield, J., and Skinner, M. K. (2006) *Reproduction 132*, 877–886.
17. Kase, H., Hattori, Y., Jojima, T., Okayasu, T., Tomizawa, A., Suzuki, K., Banba, N., Monden, T., Satoh, H., Akimoto, K., and Kasai, K. (2007) *Life Sci. 81*, 939–943.
18. Strum, J. C., Swenson, K. I., Turner, J. E., and Bell, R. M. (1995) *J. Biol. Chem. 270*, 13541–13547.
19. Takeshita, A., Shinoda, H., Nakabayashi, Y., Takano, A., Matsumoto, K., Suetsugu, M., Miyazawa, K., Tanaka, S., Endo, H., Ueyama, Y., Hanzawa, A., Suda, Y., Kanegae, H., and Yasui, T. (2005) *J. Oral Sci. 47*, 43–51.
20. Kobayashi, S. D., and Nagiec, M. M. (2003) *Eukaryotic Cell 2*, 284–294.

Downloaded by UNIV OF FLORIDA on August 14, 2009
Published on May 19, 2009 on http://pubs.acs.org | doi: 10.1021/bi9006258